Molecular pathogenesis of septicaemic and uropathogenic *Escherichia coli* and the host response to infection

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To my family
ABSTRACT

The ability to colonise and survive in the host is of major importance in microbial pathogenesis. Escherichia coli are part of the commensal microbial community in the human gastrointestinal (GI) tract. However, certain E. coli strains are capable of causing intestinal (e.g. diarrhoea) or extraintestinal diseases (e.g. urinary tract infection (UTI)). Over the past 20 years, E. coli capable of translocating through the gut epithelium to cause septicaemia have been documented. The mechanisms by which these strains translocate and interact with the gut epithelium are not fully elucidated. Furthermore, it is not clear whether septicaemic strains isolated from hospitalised patients with UTI translocate primarily from the gut (due to their prevalence in the gut) or do so via the kidneys. The overall objective of this thesis was to investigate the interaction of E. coli isolates capable of causing septicaemia and/or UTI with the human host. In the first part of this thesis, the specific aims were to investigate E. coli colonisation of the gut, translocation and the host response to infection.

For this reason, four highly efficiently translocating E. coli (TEC), isolated from the blood and/or mesenteric lymph nodes (MLNs) of man, pigs and rats were investigated for their interaction with the human gut epithelial HT-29 and Caco-2 cell lines. The host and innate immune response against TEC strains was also investigated by measuring the level of interleukin (IL)-8 produced by these and monocytic THP-1 cells. It was shown that the human TEC strain HMLN-1 adhered and translocated most efficiently followed by TEC strains originating from pigs and rats, to a lesser degree. These findings further support the hypothesis of host-species specific translocation. TEC strains were also shown to translocate by initially
adhering to microvilli, followed by intimate adhesion to cells and translocation via a cell membrane-bound vesicle. Overall, TEC strains elicited a higher IL-8 response compared to non-TEC strains. However, the highest production was elicited by rat TEC strain KIC-2 which was likely due to its immunostimulatory monomeric H21 flagellin. For comparison of colonisation ability, a collection of 70 E. coli strains from the blood of septicaemic patients without (septicaemic strains, \( n = 40 \)) and with UTI (uroseptic strains, \( n = 30 \)) were also investigated for their ability to adhere to the gut epithelium. Uroseptic strains adhered more efficiently than septicaemic strains which was most likely due to the multitude of adhesin genes that they carried.

Uropathogenic E. coli (UPEC) normally cause infection of the bladder (i.e. cystitis). However, in some cases, they may ascend to the kidneys to cause pyelonephritis, renal damage and urosepsis. It is well established that the intestinal flora is the most common source of bacteria causing UTI and the dominant strain is often the causative agent. Certain patient groups, including children and pregnant women are at higher risk of UTI. However, it is not clear whether UPEC causing UTI in children and pregnant women in different geographical areas are similar in their virulence characteristics. In the second part of the study, the prevalence of virulence genes and factors associated with colonisation and survival of UPEC were investigated among isolates from children from Australia, Iran, Slovakia, Sweden and Vietnam and from pregnant women from Sweden and Uganda. Overall, 79% of UPEC from children and 58% of those from pregnant women carried the Ag43 gene, \( flu \), suggesting a role for this virulence factor in the pathogenesis of UPEC in children. The \( flu \) gene and allelic variant genes \( fluA_{CFT073} \) and \( fluB_{CFT073} \) were associated with phylogenetic group B2 and antibiotic resistance. Prevalence was
lowest among Iranian isolates compared to those from other countries. Lack of
expression of curli and cellulose was low among all isolates and was associated with
antibiotic resistance among Vietnamese isolates. Interestingly, antibiotic resistance
was significantly high among isolates from Iran, Vietnam and to a lesser degree,
Uganda, compared to isolates from Australia, Slovakia and Sweden.

An increase in antibiotic resistance among UPEC isolates can make the treatment of
UTI more difficult. This, together with the use of traditional medicine as an
alternative treatment method among different cultures led to an investigation that
forms the last part of this thesis. The effects of the aqueous extract of a traditional
medicinal herb *Labisia pumila* var. *alata* (LPva) on the host response to UPEC
infection was investigated using a T24 cell culture model of the uroepithelium. It
was found that cells treated with LPva extract induced apoptosis without and with
UPEC infection which was coupled with upregulation of the apoptosis-promoting
caveolin-1 protein. On the other hand, non-treated cells underwent late stages of
apoptosis and necrosis in the presence of infection. Finally, treatment of T24 cells
with LPva extract was shown to decrease the level of bacterial invasion and the
expression of invasion-mediating β1 integrin compared to non-treated cells.
Collectively, these results indicated that LPva extract may have protective functions
by reducing bacterial load in the uroepithelium.
DECLARATION OF ORIGINALITY

This thesis has not been previously submitted for a degree or diploma at this or any other university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Nubia Ramos

May 2011
PUBLICATIONS RELATED TO THIS THESIS


5. Ramos, N. L., M. Sekikubo, M. Katouli and A. Brauner. Virulence characteristics associated with long-term survival of uropathogenic *Escherichia coli* in pregnant women. (Manuscript in preparation.)

OTHER PUBLICATIONS


Virulence genes of translocating *Escherichia coli* and their prevalence among septicaemic isolates of gut and urinary tract origin. Queensland Winner of the Becton Dickinson Student Award, Australian Society for Microbiology Annual Conference, Melbourne, Australia.
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Special thanks to all of my colleagues both at the Microbiology Laboratory at the University of the Sunshine Coast and at the Department of Microbiology, Tumour and Cell Biology, Division of Clinical Microbiology, Karolinska Institutet and Karolinska University Hospital, especially Drs. Fazlina Mansor and Petra Lüthje for all of their contributions, help and discussions on our projects. It was a pleasure working with you and I fondly remember fun times in and outside of the lab. I also appreciate the help and contribution provided by my fellow PhD students Tara Vollmerhausen, Emma Anastasi, Aycan Gündoğdu and Ysanne Hook at the USC.
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<tbody>
<tr>
<td>Ag43</td>
<td>Antigen 43</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
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<tr>
<td>BMECs</td>
<td>Brain microvascular endothelial cells</td>
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<tr>
<td>BPT</td>
<td>Biochemical phenotype</td>
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<tr>
<td>BT</td>
<td>Bacterial translocation</td>
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<tr>
<td>CEACAM</td>
<td>Carcinoembryonic antigen-related cellular adhesion molecule</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CNF1</td>
<td>Cytotoxic necrotising factor 1</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidine-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>ESBL</td>
<td>Extended spectrum beta lactamase</td>
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<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
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<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
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<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>ICU</td>
<td>Intensive care unit</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LPva</td>
<td><em>Labisia pumila</em> var. <em>alata</em></td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix-associated laser desorption/ionisation time-of-flight analysis</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MDR</td>
<td>Multidrug-resistant</td>
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<tr>
<td>MLNs</td>
<td>Mesenteric lymph nodes</td>
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<tr>
<td>MS/MS</td>
<td>Mass spectrometry/mass spectrometry analysis</td>
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<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
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<tr>
<td>OmpA</td>
<td>Outer membrane protein A</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute cell culture medium</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SEM</td>
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<td>TEC</td>
<td>Translocating <em>Escherichia coli</em></td>
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<tr>
<td>TEER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>UTI</td>
<td>Urinary tract infection</td>
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<tr>
<td>UPEC</td>
<td>Uropathogenic <em>Escherichia coli</em></td>
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<td>UPGMA</td>
<td>Unweighted Pair Group Method with Arithmetic Mean</td>
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CHAPTER 1

General Introduction and Literature Review

1.1  *Escherichia coli* and extraintestinal diseases

*Escherichia coli* is a facultative anaerobe that colonises the caecum and colon of the human gastrointestinal (GI) tract (223). Although other anaerobes such as *Bacteroides* spp., form bigger populations in the GI tract (47, 63), the bacterial species *E. coli* is nevertheless an important member of the intestinal commensal community (223). While generally not adversely affecting the host in this environment, under certain circumstances *E. coli* may cause intestinal and extraintestinal diseases (70, 115, 223). The virulence factors and mechanisms by which pathogenic *E. coli* cause intestinal or extraintestinal illness vary and form the basis for classification into the different pathotypes (54, 115).

Extraintestinal pathotypes are generally referred to as extraintestinal pathogenic *E. coli* (ExPEC) (199) and include meningitis/sepsis-associated *E. coli* (MNEC) and uropathogenic *E. coli* (UPEC) (115). It has been shown that *E. coli* causing meningitis in neonates, primarily *E. coli* K1 strains, are able to move across gut epithelial cells (32) and brain microvascular endothelial cells (BMECs) (121). Similarly, gut-associated *E. coli* causing a systemic inflammatory response (sepsis) in surgical patients (herein referred to as septicaemic *E. coli*) likely move through gut epithelial cells using a process known as bacterial translocation (BT) and are one of the most common enteric species at the postoperative septic foci (140, 174, 191, 192, 241). On the other hand, intestinal *E. coli* in the host faeces are the most common colonisers of the urinary tract and are responsible for the majority of
urinary tract infections (UTIs) (73, 76, 115, 198, 244). Importantly, UTIs are one of the most prevalent bacterial infections among humans (75).

The clinical manifestations of ExPEC infection can range from asymptomatic bacteriuria (75) to sepsis and death (75, 110, 205). Immunocompromised patients are at highest risk of sepsis caused by BT from the gut (141, 174). In contrast, UTI usually occurs among otherwise healthy patients (75, 76) of either gender and of any age, although incidence can be higher among certain groups (75). The ascension of UPEC to the kidneys may, in certain circumstances, cause severe renal damage (130, 144) and urosepsis (75, 105, 110).

The virulence characteristics of septicaemic E. coli capable of translocation are not well known but have been associated with increased colonisation of the GI tract preceding BT (140). Despite the severity of illness that can be caused by translocating E. coli (TEC), to date, few strains have been identified and studied, indicating the need for further research into their virulence properties. Among UPEC, high recurrence rates among UTI sufferers (75, 76) underscore the mechanisms of action, including adhesion and invasion of the host uroepithelium, which may facilitate their persistence in the urinary tract (19, 161, 162). Adhesins, including type 1 fimbriae and P pili are highly common among UPEC (36, 111) and have roles in colonisation and invasion of the uroepithelium (9, 48, 105). In particular, type 1 fimbriae-mediated invasion has been shown to occur via binding to β1 and α3 integrin protein receptors on the host cell surface (66). Less is known about the prevalence of other virulence factors associated with UPEC persistence and survival.
Although commensal bacteria positively contribute to the development of the host immune system (175), in the face of infection, epithelial cells must recognise pathogenic bacteria (12) and elicit immune responses aimed at protecting the host (136, 148). In the urinary tract, an array of host responses may be initiated against UPEC infection including the secretion of antimicrobial peptides, cytokines and chemokines and the recruitment of neutrophils (217). A significant host response to infection, which may be induced by bacterial type 1 fimbriae is programmed cell death (161). Interestingly, the expression of the membrane protein caveolin-1 may play a role in sensitising cells to apoptotic stimuli (132). While medicinal plant products may be useful in preventing UTI, from the clinical point of view, antimicrobial drugs may be taken to eradicate infection. However, increases in the prevalence of antibiotic resistance (232) indicate the need for alternative treatment practices and options.

1.2 General objectives of this thesis

In view of the above, the objectives of this thesis were to investigate the molecular pathogenesis of septicaemic and uropathogenic *E. coli*, with particular focus on mechanisms of action associated with colonisation, invasion, translocation and long-term survival, and to investigate the host response to such infection. Since BT through the gut epithelium is believed to be a mechanism of septicaemic *E. coli* (140, 174, 191, 241), a collection of TEC isolates which were found to translocate in their original hosts (man, pigs and rats) were tested for their ability to adhere and translocate in a human cell culture model of the gut epithelium. Comparisons were made to clinical isolates from patients suffering septicaemia with and without UTI.
The host epithelial and innate immune response to TEC infection was measured via the production of the chemokine interleukin-8.

Given that UPEC are often implicated in UTI and recurrent infection (76), virulence properties associated with long-term survival in the urinary tract were investigated in isolates from two high risk patient groups, i.e. children and pregnant women. Isolates from children in 5 different countries and pregnant women from 2 countries were tested for phylogenetic groups, the prevalence of antigen (Ag) 43 genes, the expression of curli and cellulose and their level of antibiotic resistance.

Finally, the effects of the Malaysian medicinal herb *Labisia pumila var. alata* (LPva) on the host response to UPEC infection were investigated. Both the health conditions for which LPva is traditionally used and its antioxidant components indicated potential effects against urinary tract infection. Therefore, the effects of LPva treatment on programmed cell death, expression of caveolin-1 and β1 integrin, and UPEC adherence and invasion were investigated to determine whether LPva had applications against UPEC infection of the uroepithelium.

The remainder of this chapter provides an overview of the literature relevant to the research undertaken.

1.3 *E. coli* in the human host

1.3.1 Microflora of the GI tract
Like many other members of the gut microflora, *E. coli* is established in the human GI tract soon after birth (115, 175) and colonises this site throughout the lifespan (223). A resident *E. coli* strain may inhabit the GI tract for long periods of time, even
several years, and once established may be predominant compared to other *E. coli*
strains that only briefly colonise this site (223, 235).

The health of the human host benefits from the presence of these and other bacteria
in the gut. The GI microflora has three main roles in the host (175). These functions
include competitive adherence to the gut epithelium to protect the host from
colonisation by pathogenic bacteria, roles in the development of the immune system
and roles in the synthesis of nutrients which the host cannot readily obtain from food
during digestion (70, 175). In particular, *E. coli* has been shown to prevent
colonisation of pathogenic *Salmonella typhimurium* in a germfree mouse model (98).

Pathogenic *E. coli* types have evolved through the acquisition of specific virulence
geness that enable their ability to cause intestinal or extraintestinal infection (54, 115).
While commensal *E. coli* may carry fewer genes than pathogenic types (54), in
certain situations the acquirement of virulence genes may enable these bacteria to
cause illness (70, 223). On the other hand, the loss of gene function may enable
pathogenic strains to survive in a new host niche (54, 124, 147). This has been
demonstrated in *E. coli* causing asymptomatic bacteriuria (124). Klemm *et al.* 2006
(124) showed that although these bacteria carry genes for adhesins associated with
symptomatic UTI (type 1 and P fimbriae), they do not express functional organelles
and thus do not induce an inflammatory response which enables their commensal-
like persistence in the host urinary tract. The development of antibiotic resistance
may also enable increased pathogenicity among the normal gut microflora (223).
1.3.2 Intestinal pathotypes

Although not the focus of this thesis, *E. coli* pathotypes causing intestinal disease are of importance to human health due to their association with a high incidence of diarrhoea in developing countries (43, 165). Types associated with intestinal disease include diffusely adherent *E. coli* (DAEC), enteroaggregative *E. coli* (EAggEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC) (115, 165). Finally, another intestinal pathotype is adherent-invasive *E. coli* (AIEC) which is associated with severe GI inflammation (17, 115). Although also present in healthy hosts, AIEC strains are more highly prevalent among Crohn’s disease patients (16). Mechanisms of action employed by intestinal pathotypes include adherence, invasion of the host mucosa and/or the production of toxins (115, 165).

Young children are the most commonly affected group suffering diarrhoea caused by DAEC (153, 207), EAggEC (96), EPEC and ETEC (159). Otherwise healthy adults may also suffer traveller’s diarrhoea caused by ETEC (115, 165). In addition to diarrhoea, other diseases that are associated with these pathotypes include ‘dysentery-like’ illness caused by EIEC, and vomiting and haemolytic uremic syndrome caused by EHEC (43, 115, 165). These strains do not colonise the host long-term but rather, cause disease when ingested (198). Contaminated food and/or water are the most common sources for most, if not all diarrhoeagenic pathotypes (165).
1.3.3 Extraintestinal pathotypes

Unlike intestinal pathotypes, ExPEC strains causing meningitis (145), sepsis (56, 140, 142) and UTI (115, 198) colonise the GI mucosa but do not normally cause disease at this site (110, 198). Rather, these bacteria possess virulence factor genes, generally carried on pathogenicity-associated islands (PAIs), whose expression enables them to cause extraintestinal infection (110). Virulence factors carried by ExPEC strains include adhesins such as F9 fimbriae (229), P pili, type 1 fimbriae and S fimbriae; toxins such as cytotoxic necrotising factor (CNF) 1 and haemolysin; polysaccharide capsule components such as lipopolysaccharide (LPS) and siderophores including as aerobactin systems (111). These virulence factors contribute to ExPEC pathogenesis in different ways (111).

Meningitis/sepsis-associated *E. coli* (MNEC), one of these extraintestinal pathotypes, predominantly causes meningitis among neonates compared to patients of other age groups (198). A recent study on *E. coli* meningitis in infants aged less than 3 months old reported a mortality rate of 14% (94) which was less than previously reported mortality rates ranging from 25–40% (25). *E. coli* K1 strains are the most common cause of meningitis (32, 145). These bacteria may be acquired during delivery, colonise the GI tract (32) and thereafter move from the gut to the meninges (25, 32, 145). Although the mechanisms employed in this latter process have yet to be fully elucidated (145), it has been shown that *E. coli* K1 is able to translocate across gut epithelial Caco-2 and T84 cells (32). This strain is also able to invade and translocate through the blood-brain barrier to cause inflammation of the meninges (121). Adhesion and invasion are facilitated by many virulence factors amongst which the
K1 capsule (122, 126), S fimbriae (126), Ibe10 protein (97) and outer membrane protein (Omp) A (186) play key roles in the pathogenicity of MNEC.

The MNEC pathotype also includes sepsis-associated *E. coli* (115). While MNEC predominantly cause meningitis in neonates and to a lesser degree among adult patients with head trauma or those who have undergone neurosurgery (198), gut-origin *E. coli* have been shown to cause sepsis among surgical patients (140, 141, 174, 191, 192, 241). Although little is known about the virulence properties of these septicaemic *E. coli*, it has been found that increased intestinal colonisation is associated with later presence at the septic foci of adult patients undergoing abdominal surgery (140, 174). Such findings indicate the translocating ability of these strains (140, 141, 174, 191, 192, 241).

The predominant causative agent of UTI is *E. coli* (73, 76, 115, 196, 198, 244). These bacteria commonly carry many virulence factors associated with adhesion (115). Furthermore, ability to invade the uroepithelium enables UPEC persistence in the host and may afford protection from antimicrobial treatment (76). In females, the introduction of host faecal matter into the vagina provides UPEC with the opportunity to colonise the urethra prior to its passage up the urinary tract to cause cystitis and pyelonephritis (73, 76, 244). Although UTI may be suffered by many different patient groups, those most susceptible include young children, women and mature-age adults (75). Among women, incidence is highest among those aged 20–56 years of age (76). Sexually active and pregnant women are high-risk groups (75). The overall high incidence of UTI is associated with significant economic cost (198).
It is evident that a number of key mechanisms may be employed in the pathogenesis of both septicaemic and uropathogenic *E. coli*. The following section therefore explores virulence mechanisms including adhesion, invasion and BT with particular focus on those associated with *E. coli* causing extraintestinal infection.

1.4 **Virulence mechanisms associated with extraintestinal infection**

1.4.1 **Adhesion**

In general, bacterial adhesion to the host mucosa must occur to enable *E. coli* to cause intestinal (136) and/or extraintestinal infection (54, 160). Adhesion is generally mediated by the binding of surface adhesins such as fimbriae or pili to host cell receptors (54, 115). *E. coli* of different pathotypes carry adhesin virulence factors which mediate their attachment to host cells including curli fimbriae, Dr fimbriae, F1C fimbriae, P pili, S fimbriae, type 1 fimbriae and OmpA (11, 115).

For UPEC, adhesion not only enables colonisation of the urinary tract but may also protect bacteria against host mechanisms which aim to eradicate infection (e.g. urine flow), may activate signalling pathways between host and bacterial cells and facilitates invasion of bladder epithelial cells (160). Figure 1.1 shows adhesion to and invasion of the host uroepithelium.
Figure 1.1 Schematic diagram of adhesion and invasion of the human uroepithelium by uropathogenic *E. coli*. Bacteria adhere to the superficial umbrella cells at the apical surface of the uroepithelium (1). Thereafter internalisation of UPEC begins via envelopment of the bacterial cell (2), UPEC invade cells in membrane-bound vacuoles in superficial cells (3). Bacterial cells may move to deeper layers of the uroepithelium (4) and may persist in the host by forming intracellular bacterial communities at this site. Adapted from Mulvey *et al.* (2000) (163).

Among UPEC strains, type 1 fimbriae and P pili play key roles in UPEC pathogenesis (9, 48, 85). FimH, at the tip of type 1 fimbriae, binds to mannose-containing glycoprotein host cell receptors such as uroplakin Ia (161, 248). Type 1 fimbriae may also be involved in other UPEC mechanisms and signalling pathways which will be discussed further on in this review. Interestingly, it has been shown that when type 1 fimbriae are expressed, P pili expression is down regulated and *vice versa* (220). The lack of expression of type 1 fimbriae is hypothesised to aid UPEC movement up the urinary tract to the kidneys (115). While P pili are also involved in UPEC colonisation of the urinary tract (242), these adhesins are generally associated with strains causing pyelonephritis (48, 103). PapG, the adhesin at the tip of P pili, binds to digalactoside receptors (115). PapG may also bind to receptors present on surfactant-like particles secreted by intestinal cells thus suggesting that P pili-
expressing UPEC may be able to colonise the GI tract (81). S fimbriae, involved in both UPEC and MNEC adherence, may also have roles in the extraintestinal spread of bacteria (160). Other virulence factors, including curli fimbriae (15) and autotransporter proteins such as antigen (Ag) 43 (230) are also involved in adhesion as well as cell aggregation and biofilm formation, properties which may all aid UPEC colonisation and survival (15, 230).

Intestinal pathotypes also use adhesion mechanisms to bind to host cells thus enabling them to cause disease. Briefly, DAEC, like UPEC, carry Afa/Dr fimbriae mediating their adhesion to a number of different host cell receptors including membrane protein type IV collagen, decay accelerating factor and carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) (212). AIEC also adhere to CEACAM receptors on intestinal epithelial cells from Crohn’s disease patients via type 1 fimbriae variants (16). EHEC and EPEC secrete their own intimin receptor which is thereafter translocated to the host cell membrane and facilitates bacterial cell adhesion (58). ETEC use colonisation factor antigen 1 fimbriae for adhesion (131). Collectively, the examples detailed here indicate the importance of adhesion to both intestinal and extraintestinal E. coli pathogenicity in the human host.

1.4.2 Invasion

Of intestinal pathotypes causing diarrhoea, only EIEC are considered to be invasive organisms (115). However, among extraintestinal types, E. coli often employ invasion in their pathogenesis. MNEC are able to invade brain microvascular endothelial cells (BMECs) to cause meningitis (97, 121) while septicaemic E. coli
briefly invade gut epithelial cells during the translocation process (4, 142). Finally, UPEC may opportunistically invade host cells after adhesion (115, 160, 162).

Invasion may be mediated by a number of different virulence factors including adhesins (27, 66, 146, 226) and toxins (27, 120). In the invasion process, pathogenic bacteria may be internalised in one of two ways (206). ‘Zippering’ entails cell membrane envelopment of bacteria while ‘triggering’ describes the induction of host cell cytoskeletal changes following bacterial binding to host receptors and the internalisation of bacteria in macropinocytic vacuoles (206). UPEC internalisation in mouse bladders appears to occur via a zipper-like process (146, 161).

Among MNEC, the binding of proteins such as Ibe (97, 121) and OmpA (186) to host receptors has been shown to be associated with *E. coli* K1 invasion of BMECs (121). BMECs may also be invaded by MNEC strains secreting the toxin CNF1. CNF1 mediates invasion by inducing host cell actin cytoskeletal rearrangement through the activation of RhoA GTPase (120). Interestingly, among UPEC, CNF1 has been shown to promote inflammation, adhesion and invasion in bladder epithelial cell lines but these effects could not be confirmed *in vivo* (91). The Hek outer membrane protein, expressed by *E. coli* K1, may also play roles in both adhesion and invasion (68, 69) by binding to proteoglycans on the host cell surface (68). Finally, the flagella of *E. coli* K1 strains may also have some roles in invasion of BMECs (179).

UPEC invasion occurs via lipid rafts of bladder epithelial cells (60) and is triggered by FimH adhesion to host cell receptors (146). FimH may bind to uroplakin Ia and
thereafter induce uroplakin IIIa cell signalling pathways which mediate UPEC invasion (226). Host cell invasion may also be promoted by FimH binding to β1 and α3 integrin receptors (66). Moreover, host cell cytoskeletal rearrangement (66, 146) associated with protein tyrosine phosphorylation, activation of phosphoinositide 3-kinase (146), focal adhesion and SrC kinases (66) may also be involved in FimH-mediated invasion (66, 146). Interestingly, as with *E. coli* K1 strains, the flagella of UPEC mediate invasion of renal collecting duct cells (182).

Following invasion, UPEC have been shown to form intracellular bacterial communities (IBCs) (see Fig. 1.1) in the mouse bladder epithelium (7, 160, 162) and in human uroepithelial cell lines (19). Furthermore, the presence of exfoliated IBCs in urine from women with cystitis indicates the occurrence of IBC formation in the human uroepithelium (197). Anderson *et al.* (2003) (7) showed that bacteria in IBCs form biofilms and are protected in ‘pod-like’ structures with a uroplakin shell. Bacteria may also survive intracellularly in quiescent states within immature bladder cells (67). Collectively, IBCs may aid long-term survival of UPEC, can be a source of recurrent infection in the host (7, 19, 160, 162) and may also contribute to the development of antibiotic resistance (23). While UPEC may form IBCs, certain strains may be internalised into gut epithelial cells but do not remain in this niche (142).

### 1.4.3 Bacterial translocation

Bacterial movement from the GI tract through the epithelial layer, to the mesenteric lymph nodes (MLNs) and further to extraintestinal sites such as the liver and spleen is defined as bacterial translocation (BT) (18). Since it was first described, the
definition of BT has been adjusted to include both viable and non-viable bacteria and their products (e.g. endotoxins) (4). The site of colonisation in the GI tract determines the various sections of the MLNs to which bacteria translocate before dissemination in the host (79).

Cell culture models form an important part of studies into BT. Continuous human epithelial colon carcinoma cells lines such as Caco-2, HT-29 and T84 are commonly used to model the human gut epithelium (203). Among other characteristics, growth in monolayers, formation of tight junctions, presence of microvilli on the apical surface and ability to induce differentiation make these cell lines suitable for many different studies of the gut epithelium including toxicity studies and infection assays (203). Such in vitro studies have shown that bacteria capable of translocation move through gut epithelial cell monolayers in a transcellular pathway (Fig. 1.2) (4, 55, 142). Bacterial cells adhere to the microvilli on the apical membrane, become internalised and then pass through the basolateral membrane (4, 142). While the transcellular pathway has been demonstrated, time-dependent variations have been documented (142, 166, 246). It is possible for commensal strains to move between cells undergoing cytoskeletal rearrangement due to induced metabolic stress following prolonged infection periods (i.e. >16 h) (166, 246). However, studies on translocating E. coli have shown that tight junction integrity remains constant throughout short (<6 h) infection periods, suggesting that while cell monolayers are not compromised, these strains are able to move through cells rather than between cells (55, 142, 246).
Figure 1.2 Schematic diagram of the passage through gut epithelial cells taken by translocating *E. coli*. Bacteria adhere to the microvilli at the apical surface (1). Thereafter bacterial cells are internalised in vacuoles (2), translocate through cells and exit via the basolateral membrane (3), before continuing to further sites in the host such as the mesenteric lymph nodes and sterile organs.

*In vivo*, conditions under which BT may occur include when there is bacterial overgrowth, gut barrier dysfunction and/or if the host is immunocompromised or immunosuppressed (174, 185, 221, 243). Clinically, BT has been shown in a case of fatal haemorrhagic pancreatitis (167), in patients undergoing abdominal aortic aneurysm repair (241), colorectal surgery (191), colonic resection (192), laparotomy (141, 174) and among colorectal cancer patients (37). Prevalence of BT among a large group of surgical patients over 13 years was reported to be 14% (141). In patients undergoing manipulation of the intestine during surgery however, BT has been shown to occur in almost 80% of patients (191). Overall, BT may be associated
with increased risk of sepsis, multiple organ failure and/or mortality (37, 56, 141, 174, 241).

In the laboratory, animal studies in mice, pigs, rabbits and rats have shown BT in conditions associated with increased gut permeability including acute pancreatitis (233), burn injury (5), cirrhosis (38), haemorrhagic shock (14, 30, 116, 168), intestinal obstruction (201), ischaemia/reperfusion (82, 119, 135), obstructive jaundice (178), total parenteral nutrition (243), peritonitis (119, 135) and starvation (168). BT has also been shown in immunosuppressed tumour-bearing rats (180). Finally, cell culture models of the human gut epithelium (32, 55, 142, 227) and BMECs (121) have shown BT of certain strains such as E. coli K1 (32, 121, 227) and E. coli C25 (55, 142).

Despite all the conditions under which BT may occur, a number of studies have shown that not all bacteria translocate with the same efficiency. Studies in gnotobiotic mice infected with single bacterial strains has shown that Gram-negative facultative anaerobes such as E. coli are significantly more efficient in translocation from the GI tract to MLNs compared to Gram-positive species while obligate anaerobes are the least efficient in this process (221). Clinically, E. coli is often the most commonly isolated species among patients in whom BT has occurred (140, 174, 241) thus further indicating its translocation efficiency. Other bacteria that have been less commonly shown to translocate in patients include Bacteroides sp., coagulase-negative staphylococcus, Enterococcus sp., Klebsiella sp., Proteus sp., Pseudomonas sp. and Staphylococcus aureus (37, 140, 174, 191, 241).
Although *E. coli* is able to translocate more efficiently than other bacteria, within the species itself, not all strains translocate at equal rates. Indeed, certain strains have been shown to be more efficiently translocating than non-pathogenic strains (116, 118, 135, 167). For instance, in a case of fatal haemorrhagic pancreatitis, of several different *E. coli* phenotypes that were found in the faeces of the patient, only one was found to be able to translocate, as evidenced by its presence in the MLNs, peritoneal fluid and blood (167). Troeger *et al.* (2007) (227) also showed differences in ability to translocate between *E. coli* strains from human cerebrospinal fluid, faecal and urine origin.

In animals, studies on BT in a rat haemorrhagic stress model identified two strains from the indigenous microflora of the host that were significantly more efficient in their ability to translocate compared to other strains (116). BT was not evident, or occurred in very low levels among rats not carrying these strains (116). Furthermore, these TEC strains were shown to be able to colonise the caecum of rats not previously carrying these strains (168). In the condition of haemorrhagic stress and starvation, these TEC strains were again able to translocate significantly more than indigenous bacteria (168). These TEC strains were furthermore found to translocate in pig models of ischaemia/reperfusion or peritonitis (135).

The mechanisms enabling BT are largely unknown. However, one recent study has shown that *E. coli* expressing α-haemolysin reduce gut barrier function through the induction of ‘focal leaks’ in gut epithelial monolayers, resulting in increased BT. Bacterial translocation can be further augmented in the presence of tumour necrosis factor (TNF) α and interleukin-13 (227). Strains that carry the *hlyA* gene but express
\(\alpha\)-haemolysin to a lesser degree are not able to translocate (227) further suggesting strain-specific ability to translocate. Another gene that has been associated with translocating *E. coli* (TEC) is the *hek* gene (142). This gene encodes outer membrane protein which has been shown to mediate *E. coli* K1 adhesion and invasion of gut epithelial cells (68). Interestingly, few of the currently known pathogenic virulence genes are carried by TEC strains (142, 227) suggesting that other as yet unknown virulence factors and genes may be involved in the pathogenesis of TEC strains.

1.5 **The host response to infection**

1.5.1 **Host-microbe interactions in the GI tract**

Bacterial adherence to host cells may trigger a cascade of cell signalling that leads to an immune response to infection (59, 160, 162). From the time of colonisation of the GI tract, interactions between host cells and commensal bacteria contribute to the development of the host mucosal immune system (12, 34, 175). Additionally, communication between the gut epithelium, immune cells and the resident microflora maintains homeostasis of the gut ecosystem which is important for the health of the host (12, 150). In the face of infection, however, the host immune defence system must also be able to differentiate between commensal and pathogenic strains and elicit an appropriate protective response (12).

Epithelial cells, lining the mucosal surfaces of the GI and urinary tracts, may be the binding site of both commensal and pathogenic strains and play a key role in maintaining immune homeostasis (12, 113, 136). As well as providing a physical barrier, epithelial cells secrete antimicrobial peptides and are also involved in detecting bacteria (12, 148, 150). If bacteria are recognised, epithelial cells initiate
and regulate inflammatory and immune responses (59, 113) which include the secretion of inflammatory cytokines and chemokines that attract other immune cells (e.g. polymorphonuclear leukocytes) to the site of infection (113). Specialised epithelial M cells are involved in the development of the host immune response by sampling and delivering bacterial antigens directly to immune cells in the gut-associated lymphoid tissues (GALTs) (12, 148, 150, 216).

The GALTs, including the appendix, tonsils, MLNs, lymphoid follicles and Peyer’s patches, form the mucosal immune system (12, 34). Immune cells of the GALTs include B cells, CD4+ and CD8+ T cells, immunoglobulin (Ig) A plasmablasts, dendritic cells, mast cells and natural killer cells (34). In the presence of pathogenic infection, innate immune cell pattern recognition receptors (PRRs) such as Toll-like (TLR) and Nod-like (NLR) receptors identify pathogen-associated molecular patterns (PAMPs) and trigger signalling pathways leading to innate and adaptive immune responses through the production of proinflammatory cytokines and chemokines (12, 59, 154). Flagellin protein is an example of a PAMP which may be detected by TLR5 or the NLR Ipaf, depending on whether it is found extracellularly or intracellularly, respectively (154).

As indicated above, while ExPEC may colonise the GI tract, they do not normally cause disease at this site (110, 198), perhaps making it difficult to measure an immune response to these strains in a healthy host. However, a number of studies have investigated the immune response when BT has occurred. Interestingly, somewhat contradictory findings regarding the gut immune response in the presence of BT and sepsis have been reported. Coutinho et al. (1997) (52) showed that the
tissue from patients with intra-abdominal sepsis had reduced levels of IgA and IgM plasma cells compared to control samples from transplant donors and postulated that this decreased level in immune function may promote BT. On the other hand, Woodcock et al. (2001) (240) showed that in tissue from small bowel resection patients in whom BT was confirmed, an elevated host immune response indicated by increased levels of IgA and IgM positive plasma cells, was observed. In animal studies where decreased gut barrier function has been induced with total parenteral nutrition, it has been found that this treatment promotes BT, coupled with reduced host immune function (243). As can be seen, differences in the immune response to BT have been reported which may be associated with the limitations of the origins of tissue samples and/or the animal model used (240). Collectively, these data indicate the need for further research into the effects of BT on the host immune response.

1.5.2 Defence mechanisms of the urinary tract

The urinary tract is equipped with a multitude of defence mechanisms that aim to protect against infection (40, 217, 247). Although itself generally a sterile environment (163), its close proximity to the rectum and rectal flora increases the risk of bacterial colonisation (40). The urinary tract presents an impermeable barrier for uropathogens consisting of multiple layers of transitional epithelium overlaying the lamina propria (40, 163). In the event of bacterial infection, one of the first lines of defence is the flow of host urine (40, 163). Indeed, urine flow may remove and/or prevent bacterial adherence to the uroepithelium while its chemical properties and components, including pH, osmolarity and urea, may affect bacterial ability to survive (163). However, bacteria expressing type 1 fimbriae may overcome this host defence mechanism as the shear stress of urine flow has been shown to increase
FimH-mediated adhesion to mannose-containing surfaces (225). Other components that are secreted into the urine, including Tamm-Horsfall protein and secretory IgA, may also protect the urinary tract by preventing bacterial adherence (40, 163). Despite the potential protective effects of urine however, uropathogens may yet colonise the uroepithelium thus requiring the action of other host immune responses (40, 163, 217).

Many defence mechanisms employed in the GI tract are also employed in the urinary tract. Renal epithelial cells have been shown to express proinflammatory cytokines such as IL-1α, IL-1β and TNF-α in response to UPEC infection (28). Other immune components employed in the urinary tract include chemokines, complement, phagocytic cells (e.g. dendritic cells, macrophages and neutrophils), lymphocytes and adaptive cells (217). Additionally, like the gut epithelium, the uroepithelium contains PRR receptors which recognise FimH, flagellin and LPS on UPEC surfaces and induce innate and adaptive immune responses upon their detection (217). LPS binding to TLR receptors activates nuclear factor-κB, coupled with increased IL-6 production in human dermal endothelial cells (71). In the uroepithelium, FimH activates IL-6 production in an LPS-dependent pathway (208), binds directly to TLR4 receptors and also induces the production of proinflammatory cytokines (e.g. tumour necrosis factor (TNF)-α and IL-8) (158), and antiviral responses (i.e. the production of interferons) (13). As well as the activation of proinflammatory cytokines, FimH may also induce the exfoliation of superficial bladder epithelial cells (161). While this host response may be aimed at eradicating bacterial infection from the mucosa, it may, on the other hand, provide UPEC with the opportunity to invade deeper layers of the uroepithelium (161).
Like epithelial cells in the GI tract, uroepithelial cells express antimicrobial peptides such as α and β defensins and cathelicidin (LL-37) as part of the immune defence (217, 247). In the case of LL-37, it has been shown that expression is significantly increased during UTI which functions to protect the uroepithelium from UPEC invasion (41). The antimicrobial proteins lactoferrin and lipocalin disrupt iron homeostasis in bacteria as part of the innate defence system (247). Many other factors are associated with the host response to infection including cytoskeletal components (e.g. actin), enzymes (e.g. Rho GTPases) and kinases (e.g. focal adhesion kinase) which are all involved in UPEC internalisation as well as proteins (e.g. MyD88) involved in proinflammatory gene expression (217).

Despite all the mechanisms that may be employed to protect the host, bacteria may still survive to cause sepsis and/or UTI. The following section therefore explores the pathogenesis of these two disease processes.

1.6 Pathogenesis of extraintestinal disease

1.6.1 Sepsis

Sepsis is the term used to define the ‘systemic inflammatory response to infection’ and is characterised by disruption of homeostasis in the host (26). Clinical conditions that may be seen among septicaemic patients include the presence of infection in the bloodstream, increased or decreased body temperature (>38°C or <36°C), increased heart rate (>90 beats/min) and respiratory rate (>20 breaths/min) and severe changes in white blood cell count (<4000/mm³ or >12 000/mm³) (26). Septicaemic patients may develop severe clinical conditions which can include organ dysfunction,
hypoperfusion, hypertension or septic shock (26). Sepsis is also characterised by increased production of cytokines which drive the inflammatory processes (22, 26, 87). As described above, bacterial surface components such as FimH (13, 158, 208), LPS (71, 88) and curli fimbriae (20) stimulate cytokine production. In the condition of sepsis, cytokines are released in different stages which are collectively known as the ‘cytokine cascade’ (22, 87). The first to be released include TNFα and IL-1α and -1β (22). These trigger the release of other cytokines such as IL-6, which activates B- and T- lymphocytes, and IL-8 which is neutrophil-attracting (22). To counteract the pro-inflammatory response, anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 may also be released (26). Despite the protective effects of cytokines however, excessive production can be damaging to host tissues and can thus pave the way for multiple organ dysfunction which is the end stage of sepsis (22, 26, 87).

Many different organisms can be the causative agent of sepsis. Of these, *E. coli* is the most commonly identified enteric species (74, 127, 140). Other organisms that may cause sepsis include *S. aureus* (74, 127), *Enterococcus* sp., *Pseudomonas* sp., *S. epidermidis*, *Streptococcus* sp., *Candida albicans*, *Proteus* sp., *Bacteroides* sp., and *Klebsiella* sp., among others (74, 127, 140). Infection at many different sites including cardiac, blood, bone, intra-abdominal, intravenous catheter, pulmonary, skin and urinary sites can lead to sepsis in the human host (74). The ‘gut origin of sepsis’ hypothesis suggests that intestinal microflora employ BT to move through the gut epithelium and cause sepsis in the host (56, 174). Clinically, the gut is an important source of sepsis with a number of studies confirming it to be the origin of sepsis among surgical patients (140, 141, 174, 191).
Sepsis is associated with significant mortality rates and medical and economic burden (10). The prevalence of severe sepsis among Australian and New Zealand intensive care units (ICUs) was found to be 0.77 per 1000 of population in 2004 (74) while in the United States of America, incidence was more than 3 times higher (3.0 per 1000 population) in 2001 (10). According to Finfer (2004) (74), 12 out of 100 ICU admissions developed severe sepsis in Australia which is similar to the reported 16% in surgical ICUs in the USA (183). The reported mortality rates among patients suffering severe sepsis vary between countries. For example, the mortality rate in Australian and New Zealand ICUs is reported to be 37.5% (74) compared to 52% in Italy (202). Septic shock is associated with mortality rates as high as 82% (202). The severe nature of sepsis and the high incidence of infection caused by *E. coli* suggest the need for further investigation into the mechanisms of action of these bacteria.

1.6.2 Urinary tract infection

As indicated in other sections of this review, UTI occurs following bacterial adhesion and colonisation of the uroepithelium (144, 160). UTI can be uncomplicated (i.e. not associated with the presence of anatomical disorders or clinical instrumentation), complicated (i.e. associated with urological abnormalities) and may be symptomatic or asymptomatic (75, 198). UTIs are classified based on the site of the urinary tract at which the infection is located (75). This may include the bladder (cystitis), kidney (pyelonephritis), prostate (prostatitis) or urine (bacteruria) (75). Progression of disease may lead to renal damage (130, 144), urosepsis and possible mortality (75, 198). The presence of bacteria in the urine (>10⁷ CFU/ litre), together with at least one other symptom is used to diagnose infection (231). These symptoms include pain during urination (dysuria) and/or
increased frequency of urination (75, 76, 112, 231), fever (>38°C), flank pain and/or lumbar pain (231). Upper UTI may be associated with additional symptoms such as general malaise and blood in the urine (haematuria) (76). Urinalysis showing positive urinary leukocyte esterase and pyuria (≥10⁷ white blood cells/high-power field; nonspun) may further confirm UTI (231).

In females, the urinary tract is anatomically in close proximity to the vagina and thus to bacteria such as *Lactobacillus* spp., *Streptococcus* spp., *Staphylococcus* spp. and *Bacteroides* spp. which colonise this site (84, 100). However, faecal organisms may also come to colonise this area and may subsequently cause UTI (73, 76, 115, 196, 198, 244). Different bacteria may be responsible for causing uncomplicated or complicated infections (196). Those causing uncomplicated UTI include *E. coli*, *Klebsiella* spp., *S. saprophyticus*, *Enterococcus faecalis* (196) and Group B Streptococcus (231). In addition to these, organisms that may cause complicated UTI include *Enterobacter cloaca*, *Serratia marcescens*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and Group B streptococci (196). Of these, *E. coli* is the most common causative agent, accounting for 80–95% of all UTIs (76, 196, 198) and 78% of recurrent infections (76).

UTI is one of the most prevalent bacterial infections among humans (75, 144, 198) and may annually be suffered by up to 175 million people worldwide (198). In the USA, up to 8 million people suffer UTI per year, generating significant economic burden (95, 198). Prevalence is highest among certain patient groups including young children, women and the elderly as well as patients with medical conditions such as multiple sclerosis and spinal cord injuries (75). A number of factors have
been identified as predisposing patients to UTI including genetic and biological factors as well as behavioural practices (75, 196). Particularly among women, sexual intercourse, the use of contraceptive measures such as diaphragms and spermicides, previous antibiotic use (75, 76, 196) and changes in oestrogen levels (190) significantly increase the risk of UTI and its recurrence.

Despite the wide body of knowledge on the virulence factors of UPEC, these bacteria continue to cause UTI far more often than any other species thus underscoring the need for better understanding of their virulence properties. Research in this area may enable more targeted treatment options. This is of increased importance in view of the increasing incidence of antibiotic resistance that has been reported among uropathogenic bacteria (86).
CHAPTER 2

Septicaemic *E. coli*: gastrointestinal colonisation, translocation and the host response to infection

2.1 Introduction

Sepsis is a serious medical condition which can result in multiple organ failure and even death (26, 56). It has been shown that bacteria of the gastrointestinal (GI) microflora are often the source of sepsis in surgical patients, a phenomenon described by the “gut origin of sepsis hypothesis” (56, 140, 191, 241). Bacterial translocation (BT) is one of the main causes of sepsis and is defined as the passage of viable bacteria and/or their products through the intestinal epithelium to the mesenteric lymph nodes (MLNs) and further to normally sterile sites (4, 18, 140). Among surgical patients, bacterial colonisation of the upper GI tract increases risk of BT (140) while overgrowth promotes its occurrence (174). Although *E. coli* forms a small population in the intestinal microbial community, making up less than 0.5% of the faecal flora (156), it is one of the most commonly isolated species at the septic foci of surgical patients in whom BT has occurred (37, 140, 174, 191, 241).

Numerous studies in animals have demonstrated that BT is associated with increased gut permeability (4, 14, 30, 38, 82, 116, 119, 168, 201, 221, 233). However, even in such conditions, not all bacterial species translocate with the same efficiency (174, 221). Indeed, Gram-negative facultative anaerobes are more efficient in BT than Gram-positive and obligate aerobe species (221). The clinical finding that *E. coli* originating from the gut is one of the most common species employing BT and
sepsis further highlights the importance of these bacteria in this process (37, 141, 191, 241).

Few translocating *E. coli* (TEC) strains have been isolated and studied. Among these, four highly efficiently TEC strains have been documented including strains isolated from the blood of humans (TEC strain HMLN-1) (167), pigs (TEC strain PC-1) (119) and rats (TEC strains KIC-1 and KIC-2) (14). A common feature of these TEC strains has been their ability to translocate more efficiently than other indigenous bacteria (including other *E. coli*) in the host (14, 119, 167). Furthermore, it has been shown that when rat-origin TEC were orally inoculated into rats lacking these strains, the inoculated strains were able to colonise and translocate in the new host, thus confirming this unique ability (168). Collectively, findings from the above-mentioned studies have led researchers to question whether strains capable of translocation in their original host were also able to translocate in other hosts.

To investigate this, Katouli *et al.* (2009) (119) tested TEC strains from human, pigs and rats for their ability to translocate in the original and other hosts. For instance, the human TEC strain HMLN-1 was tested for its ability to translocate in Caco-2 cells which is an accepted model of human gut epithelial cells, in a pig model of peritonitis/sepsis and ischaemia/reperfusion and in a rat model of starvation (119). Similarly, pig TEC strain PC-1 and rat TEC strains KIC-1 and KIC-2 were tested in each of these models. It was shown that TEC strains were most efficient in their own host-origin model compared to strains of other origins (119). It was also found that although pig TEC strain PC-1 was also able to translocate in rats to a much lesser degree than the rat TEC strains KIC-1 and KIC-2, human strain HMLN-1 did not
translocate in any of the rats tested (119). These findings clearly indicate that translocation efficiency of *E. coli* strains is host-species specific (119).

Until recently, the virulence profiles of these TEC strains were unknown. In a previous study by this author, these four TEC strains were screened for the presence of 47 virulence genes associated with pathogenic *E. coli* causing intestinal and/or extraintestinal infections (187). The results indicated that TEC strains did not carry many of the known virulence genes commonly found among pathogenic *E. coli* strains except that all strains carried the adhesin genes *fimH* and *bmaE* with HMLN-1 and PC-1 strains also carrying the capsular synthesis gene *kpsMT III* (187). Furthermore, the rat TEC strain KIC-2 also carried the toxin gene EAST1 (187).

Contrary to TEC strains, strains isolated from septicaemic patients with UTI (termed uroseptic strains) or without UTI (termed septicaemic strains) carried a multitude of virulence genes (187). In particular, a higher prevalence of *pap* genes was observed among uroseptic strains compared to septicaemic ones (187). For easy reference, the prevalence of virulence genes among these clinical strains is detailed in Appendix A (187). Strains from septicaemic and uroseptic patients predominantly belonged to phylogenetic group B2 (Appendix A) (187). On the other hand, TEC strains HMLN-1 and PC-1 belonged to group D and rat TEC strains belonged to phylogenetic group B1. Since TEC strains only carried few virulence genes such as adhesin and capsular synthesis genes, it is possible that these may be involved in the translocation process and/or that other, as yet unknown, genes are enable this unique ability.

Beginning with adhesion to host cells, bacteria employ many different mechanisms to colonise and survive in the host (54, 136, 160). However, adhesion itself may stimulate a protective host immune response against infection by the innate and
adaptive immune systems (59, 136, 160, 162). In this respect, epithelial cells play key roles in initiating and regulating inflammatory and immune responses (59, 113). Their secretion of inflammatory cytokines and chemokines attract immune cells such as monocytes to the site of infection (113). As precursors of macrophages and dendritic cells, monocytes are important components of the immune system and may themselves secrete proinflammatory cytokines in response to infection (211).

2.2 Aims of this study

It has been shown that bacterial overgrowth in the gut promotes BT (140, 174), however, it is not clear whether TEC strains are more efficiently adhering than other bacteria and whether this is associated with their passage between epithelial cells or via membrane-bound internalisation. The first aim of this study was to investigate the ability of a collection of TEC to interact with human gut epithelial cells. This included their adhesion and translocation across the human gut epithelium and the pathway employed in this process. Comparisons were made against a collection of non-TEC strains. In the face of infection, components of the GI tract, including the gut epithelium and immune cells may initiate host immune responses against bacteria (12, 150). To date, however, the host immune response to TEC has not been investigated. In view of this, the second aim of this study was to investigate the host response to TEC by measuring the IL-8 produced by epithelial and monocytic cells following infection with these bacteria. Different components of the bacterial surface may be targeted by the host immune response (234). The third aim of this study was therefore to investigate if the flagellin types carried by TEC strains were associated with the IL-8 response observed among epithelial and monocytic cells.
2.3 Materials and methods

2.3.1 Bacterial strains

Four efficiently TEC strains, isolated in pure culture from man (strain HMLN-1), pigs (strain PC-1) and rats (strains KIC-1 and KIC-2), were investigated in this study. Table 2.1 details the sources of each strain. Two non-TEC strains from rats (strain 46-4, shown to be capable of adhering to the rat caecum but not able to translocate, and strain 73-89, a non-adhering, non-translocating strain), were included as controls (Table 2.1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Description of source</th>
<th>Phylogenetic group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMLN-1</td>
<td>Human</td>
<td>Isolated from MLNs*, blood and peritoneal fluid of a patient with fatal haemorrhagic pancreatitis.</td>
<td>D</td>
<td>167</td>
</tr>
<tr>
<td>PC-1</td>
<td>Pig</td>
<td>Isolated from MLNs and blood of pigs subjected to ischemia/reperfusion or peritonitis.</td>
<td>D</td>
<td>119</td>
</tr>
<tr>
<td>KIC-1</td>
<td>Rat</td>
<td>Isolated from MLNs of rats subjected to 24 or 48 h of starvation with and without haemorrhagic shock.</td>
<td>B1</td>
<td>14</td>
</tr>
<tr>
<td>KIC-2</td>
<td>Rat</td>
<td>Isolated from MLNs of rats subjected to 24 or 48 h of starvation with and without haemorrhagic shock.</td>
<td>B1</td>
<td>14</td>
</tr>
<tr>
<td>Non-TEC controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46-4</td>
<td>Rat</td>
<td>Isolated from the caecal epithelium of rats subjected to 48 h of starvation with haemorrhagic shock.</td>
<td>B1</td>
<td>118</td>
</tr>
<tr>
<td>73-89</td>
<td>Rat</td>
<td>Isolated from the caecal contents of rats subjected to 48 h starvation with haemorrhagic shock.</td>
<td>B1</td>
<td>118</td>
</tr>
</tbody>
</table>

*MLNs: mesenteric lymph nodes.
In addition, 70 clinical strains collected from the blood of septicaemic patients at a tertiary hospital in Brisbane, Australia were also tested for adhesion to the human gut epithelium. Of these, 30 strains were isolated from the urine of septicaemic patients with UTI (uroseptic strains). The remaining isolates (n = 40) were cultured from the blood of patients without UTI (septicaemic strains). Negative urine cultures confirmed the absence of UTI among these patients. All bacterial isolates were obtained from blood and urine samples taken before the commencement of antibiotic therapy.

All bacterial strains were grown in Luria-Bertani (LB) broth, to log phase (3–4 h), at 37°C with agitation (100 strokes per min). The cultures were centrifuged and the supernatant discarded. Thereafter, the pellet was resuspended in phosphate-buffered saline (PBS) and the optical density (600 nm) was measured by spectrophotometer. The suspension was re-adjusted to a concentration of $10^9$ CFU/ml using a previously established optical density curve of different bacterial concentrations. The bacterial suspensions were serially diluted to the appropriate concentration before infecting cells.

To test immune response to TEC flagellin, a positive control strain, *Salmonella typhimurium* (237) was used. For this purpose, bacteria were cultured as described in section 2.3.7.

### 2.3.2 Biochemical fingerprinting and serotyping

TEC and non-TEC strains were typed with a high resolution biochemical fingerprinting method (PhPlate-RE, PhPlate AB, Stockholm, Sweden), as previously
described (117) and were serotyped at the Robert Koch Institute, Werningerode, Germany. For biochemical fingerprinting, 96-well PhP-RE plates containing 8 sets of 11 different dehydrated reagents were prepared by adding 325 µl/well of the growth medium in the first column containing no reagent and 150 µl to each of the remaining wells of the plate. The growth medium contained 0.011% w/v bromothymol blue and 0.1% w/v proteose peptone. A sample of overnight culture of each strain, grown on nutrient agar, was inoculated in each well of the first column. A 25 µl sample of this suspension was then transferred to each well along the same row. Plates were then incubated in a humidified environment at 37°C. After 7, 24 and 48 h, plates were read by scanning each using a HP Scanjet 4890 scanner. The images were then uploaded to the PhP software (PhPWin4.2) which converted the colour of each well into an absorbance value, multiplied by 10 to yield scores ranging from 0 to 30. The mean value of all readings for each strain was then calculated to create the biochemical fingerprint made of 11 scores ranging from 0 to 30 for each strain. Similarity among the strains was calculated after pair-wise comparison and the generated similarity matrix was clustered according to the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) to yield a dendogram (218). An identity level of 0.975 was established based on the reproducibility of the system after testing 10 isolates in duplicate. Those showing similarity to each other above the identity level were regarded as identical and assigned the same biochemical phenotype (BPT). BPTs containing more than one strain were called common (C) BPTs and those with one strain were named as single (S) BPTs. All data handling, including calculations of similarity coefficients as well as clustering were performed using the PhPlate software version 4002 (PhPlate AB).
2.3.3 Cell culture

Two human epithelial colorectal adenocarcinoma cell lines, Caco-2 (ATCC HTB37) and HT-29 (ATCC HTB-38) were used to model the human gut epithelium and investigate the ability of TEC strains to adhere, translocate and elicit an immune response. Cells were routinely grown at 37°C in a humidified incubator with 5% CO₂. Caco-2 cells were grown in Dulbecco’s Minimum Essential Medium (DMEM) supplemented with 20% foetal bovine serum (FBS). HT-29 cells were grown in 1640 Roswell Park Memorial Institute (RPMI) medium with 2 mM L-glutamine supplemented with 10% FBS. At confluence, cells were trypsinised using 0.05% Trypsin-EDTA. For the adhesion assay, the detached cells were resuspended in 2 ml of fresh medium, diluted 1:25 in fresh medium and then aliquoted into 24-well plates (Costar, Corning, NY, USA) containing sterile glass coverslips (Nunc, Denmark).

For the translocation assay, a suspension containing approximately $1 \times 10^5$ cells/ml was prepared before cells were seeded into the two-compartment translocation assay system (See Translocation assay, Section 2.3.4).

A human monocytic cell line, THP-1 (ATCC TIB-202), was also used to test the immune response to TEC strains. THP-1 cells were grown in suspension in RPMI 1640 medium containing 1.5 mg/ml sodium bicarbonate, 4.5 mg/ml glucose, 10 mM HEPES, and 1 mM sodium pyruvate supplemented with 0.05 mM 2-mercaptoethanol (90%). All cell culture media were renewed every other day.

2.3.4 Adhesion and translocation assays

For the adhesion assay, Caco-2 and HT-29 cells were grown to semi-confluence on glass coverslips as described above. On the day of the experiment, complete medium was removed from wells, cells were washed with PBS and 900 µl of serum-free
medium was replaced. A 100 µl volume of bacterial culture (approximately $10^8$ CFU/ml) was added to wells. Infected cells were incubated at 37°C in a humidified incubator with 5% CO$_2$ for 90 min. Following incubation, medium was removed and cells were washed three times with PBS to remove non-adhered bacteria. Cells were air-dried and thereafter fixed with 95% ethanol for 10 min. Cells were then Gram-stained, mounted onto glass microscope slides and viewed by light microscopy. The number of bacteria that had adhered per cell to a total of up to 100 randomly selected cells was counted. These counts enabled calculation of the mean number of adhered bacteria per cell and the standard error of the mean (SEM) for each strain. Assays were performed in duplicate. Clinical septicaemic and uroseptic strains were also tested for their ability to adhere to HT-29 cells, in comparison to TEC strain HMLN-1, as described above.

The ability of TEC strains to translocate across the gut epithelium was investigated using a previously described two-compartment system (55) as shown in Figure 2.1.
**Figure 2.1.** A schematic diagram showing the two compartment system used to test the translocation ability of *E. coli* strains across a cell culture model of the human gut epithelium. The bacterial inoculum was delivered into medium in the apical chamber. After the infection period, samples were collected from the medium in the basolateral chamber and were used to calculate the amount of translocation through the intact Caco-2 or HT-29 cell monolayer.

Caco-2 or HT-29 cells were seeded (1x 10^5 cells/ml) and grown to confluence in 0.8 μm pore size Millicell filter inserts (Millipore, USA) placed in 24-well plates. One empty insert (containing medium only) was maintained alongside and served as a blank control for measurement of electrical resistance generated by the insert itself. Cells were maintained as normal and the transepithelial electrical resistance (TEER) of the monolayer was measured daily using the Millicell-ERS volt-ohm meter (Millipore). The TEER, which indicates the integrity of the tight junctions (55), was calculated using the equation:

$$\text{TEER} \ (\Omega \ \text{cm}^2) = \frac{\text{measured monolayer resistance} - \text{measured blank resistance (insert lacking cells)}}{\text{area of the filter (0.6 cm}^2)} \times 0.6 \ \text{cm}^2$$

When a monolayer had formed and stable TEER readings were reached, the growth medium from the inner and outer well was removed. Cells were washed with PBS
and then supplemented with serum-free medium. Bacteria (approximately $10^6$ CFU/ml) were inoculated into the inner well of the system (Fig. 2.1). Infected cells were then incubated for 15, 30, 60 and 120 min at 37°C. At each time point, TEER readings were taken and a sample from the outer well was collected. Samples were serially diluted and 100 µl of each dilution was spread on MacConkey No. 3 agar. Following overnight incubation at 37°C, colony counts were taken to determine level of translocation for each strain.

2.3.5 Transmission electron microscopy

Caco-2 cells grown in Millicell well inserts (Millipore) and infected as per the translocation assay (see Section 2.3.4) were prepared for transmission electron microscopy (TEM) as previously described (219, 224). Following removal of medium, well inserts were fixed with 3% glutaraldehyde fixative in 0.1 M cacodylate buffer (pH 7.3) and were stored at 4°C. For TEM analysis, samples were post-fixed with 1% osmium tetroxide for 45 min and then uranyl acetate for 20 min. To dehydrate samples, each was washed in sequence with 50, 70 and 90% ethanol followed by 90 and 100% acetone. Thereafter, samples were embedded in Spurr epoxin resin, cut into sections of 50–100 nm thickness and stained with uranyl acetate and lead citrate. Preparations were viewed and photographed using a JEOL 1200EX transmission electron microscope.

2.3.6 IL-8 production in response to TEC infection

A modified method to that previously described by Chromek et al. (2005) (42) was used to test the immune response to TEC infection. Briefly, Caco-2 and HT-29 cells were seeded into 24-well plates and grown to confluence. On the day of the
experiment, medium was changed to serum-free medium. THP-1 cells were seeded into 24-well plates at a concentration of $1 \times 10^6$ cells per well in serum-free medium prior to infection. All cells were treated with gentamicin (50 μg/ml) to prevent bacterial overgrowth during the infection period. Bacteria ($10^8$ CFU/ml) were inoculated into triplicate wells and plates were thereafter incubated at 37°C in 5% CO₂. Samples of medium were collected from wells at 0, 2, 4, 6 and 24 h. For each cell line, wells containing non-infected cells were maintained in parallel as controls. Collected samples were centrifuged (300 g, 10 min, 4°C) to remove bacteria. The supernatant was stored at –80°C before performing the enzyme-linked immunosorbent assay (ELISA).

2.3.7 IL-8 production in response to purified flagellin of TEC strains

Purification of monomerized flagellin from TEC and non-TEC strains and the control strain S. typhimurium was performed as previously described (237). Briefly, TEC strains were cultured overnight in LB broth with agitation (200 strokes per min) at 37°C. Thereafter, a sample of this culture was inoculated into fresh medium (1:1000) and again incubated overnight under the same conditions. The culture was centrifuged (10 000 x g, 4°C, 20 min) and the supernatant discarded. The pellet was resuspended in 0.5 Tris-Cl (pH 8.0), then passed through a syringe to shear off flagella. Following centrifugation (8000 x g, 4°C, 20 min), the supernatant was filtered through a 0.22 μm filter. The suspension was again centrifuged (106 000 x g, 4°C, 60 min) and the pellet resuspended in PBS. Flagellin purity was analysed by 12% SDS-PAGE gels. The identity of flagellin was verified by MALDI-TOF and MS/MS analysis. To prepare monomeric flagellin, samples were adjusted to pH 3.5 using 3M HCL for 5 min at room temperature and then returned to neutral pH using
10M NaOH. Flagellin was analysed in 12% acrylamide gels with colloidal Coomassie staining.

Sequencing of flagellin was performed using primers up- and down-stream of the \textit{fliC} gene including Agstart1: ATTAGTG GGTGAATGAGGG, Agstop1: ACAA-GTCATTAATACCAACAGCC and Agstop2: GACTCCAGCGATGAAATA, as previously described (237). Additionally, primers KIC-2start: CTGGATAG-AAGAAAGATCGC and KIC-2stop: CGCTCGAAACTATCGACAAA were used to sequence TEC strain KIC-2 \textit{fliC}. Where protein sequencing revealed a high level of alignment (more than 99\%) in the sequence of the flagellin, strains representative of each H antigen subtype were selected for further testing.

To test the host immune response to secreted flagellin, HT-29 cells, grown to confluence in 24-well plates, were co-incubated with serial dilutions of monomeric flagellin diluted in RPMI for 5 h. Supernatant was then collected from wells and samples were stored at \(-20^\circ\text{C}\) for ELISA testing.

### 2.3.8 ELISA

The concentration of IL-8 produced by Caco-2, HT-29 and THP-1 cells in response to TEC infection was measured using a human CXCL8/IL-8 ELISA kit (DuoSet™ ELISA Development system, R&D Systems Inc, Minneapolis, MN, USA), as per manufacturer instructions. Briefly, 96-well microplates coated with 100 \(\mu\text{l}/\text{well}\) of capture antibody diluted in PBS were incubated overnight at room temperature. Wells were then washed three times with wash buffer. Block buffer (300 \(\mu\text{l}\)) was added to each well and plates were incubated for 1 hour at room temperature. Wells were washed and 100 \(\mu\text{l}\) of sample or standard, diluted in reagent diluent (0.1\% BSA,
0.05% Tween 20 in Tris-buffered saline), was added per well. Plates were incubated for 2 h at room temperature. Wells were washed and 100 µl of detection antibody, diluted in reagent diluent was added to each well. Plates were again incubated for 2 h, and then washed with wash buffer. Diluted Streptavidin-horseradish peroxidase (HRP) (100 µl/well) was added; plates were covered to prevent exposure to light and were incubated for 20 min at room temperature. Wells were again washed and 100 µl of substrate solution was added for 20 min. Finally, stop solution (50 µl/well) was added and the optical density of each well was measured at 540 nm. A standard curve was generated to determine the amount of IL-8 produced in response to each strain.

To measure the concentration of IL-8 produced by HT-29 cells after co-incubation with purified flagellin, a human IL-8 Eli-pair kit (Nordic BioSite AB, Stockholm, Sweden) was used according to manufacturer guidelines. Briefly, 100 µl of capture antibody diluted in coating buffer was added to each well and incubated overnight at 4°C. Wells were then washed twice with PBB-Tween (0.05%) and blocked with saturation buffer (250 µl/well) for 2 h at room temperature. Buffer was removed and plates were allowed to dry (24 h, room temperature). Standards and samples were diluted in standard diluent buffer and 100 µl of each was inoculated into wells. Thereafter, 50 µl of reconstituted biotinylated anti-IL-8 in biotinylated antibody diluent buffer was added per well and plates were incubated for 3 h at room temperature. Wells were washed with wash buffer and 150 µl of 1:100 HRP-Streptavidin was added to each well. Plates were incubated at room temperature for 20 min and then washed. Tetramethylbenzidine reagent solution (100 µl/well) was added and wells were incubated for 15 min in the dark. Finally, 100 µl of stop
solution (1M H$_2$SO$_4$) was added. The absorbance was read by spectrophotometer at 450 nm.

2.3.9 Statistical analysis

One-way analysis of variance (ANOVA) and Tukey’s Honestly Significant Difference test for multiple comparisons were used to compare the significance of difference of adhesion, translocation and IL-8 production between TEC strains. To compare the overall differences between TEC and non-TEC, and between septicaemic and uroseptic strains, unpaired t-tests with Welch correction were performed. Differences were deemed statistically significant if $P < 0.05$.

2.4 Results

2.4.1 Biochemical phenotypes and serotypes of TEC

Biochemical fingerprinting and serotyping divided the TEC and non-TEC strains into two common and two single BPTs and four different serotypes (Fig. 2.2). The TEC strains HMLN-1 and PC-1 had identical BPT (C1) and serotype (077:H18). Contrary to that, rat TEC strains KIC-1 and KIC-2, belonged to different BPTs (C2 and S1) and serotypes (O163:H14 and O102:H21, respectively). TEC strain KIC-1 had identical BPT and serotype to non-TEC strain 46-4.
Figure 2.2. A UPGMA dendrogram showing similarities between biochemical phenotypes (BPTs) and serotypes of translocating *E. coli* isolated from human (HMLN-1), pigs (PC-1), and rats (KIC-1 and KIC-2) and two non-TEC strains (46-4 and 73-89). ID-Level: Identity level above which strains were regarded as identical. Ont: O group non-typable.

2.4.2 Adhesion

The ability of TEC strains to adhere to the gut epithelium was investigated and compared to that of non-TEC controls. In the Caco-2 model, it was found that TEC strains HMLN-1, PC-1 and KIC-2 adhered to cells significantly more ($P < 0.0001$) more than TEC KIC-1 and non-TEC controls (Fig. 2.3). Overall, TEC HMLN-1 was the most adherent strain (Fig. 2.3).
Figure 2.3. The number of translocating *E. coli* adhering to Caco-2 cells after 90 min infection compared to non-translocating controls. Data are mean ± SEM from duplicate experiments.

To verify results observed in Caco-2 cells, the ability of TEC strains to adhere to HT-29 cells was also investigated. Results showed a similar pattern of adherence among HT-29 cells (Fig. 2.4); however, the mean number of adhering bacteria was lower than that seen among Caco-2 cells (see Fig. 2.3).
**Figure 2.4.** The number of translocating *E. coli* adhering to HT-29 cells after 90 min infection in comparison to non-translocating controls. Data shown are mean ± SEM from duplicate experiments.

Light microscopy showed that TEC strains adhered to both Caco-2 and HT-29 cells in a diffuse adherence pattern while a lack of adhesion was evident among non-TEC controls (Fig. 2.5).
Figure 2.5. Adhesion of translocating *E. coli* strain HMLN-1 and non-translocating control strain 73-89 to Caco-2 and HT-29 cells. (A) High degree of adhesion of TEC HMLN-1 to Caco-2 cells was observed compared to (B) sparse adhesion of non-TEC strain 73-89. (C) Adhesion of TEC HMLN-1 to HT-29 cells compared to (D) lower degree of adhesion of non-TEC strain 73-89. Images shown are Gram-stained bacteria representative of results seen for TEC HMLN-1, PC-1, and KIC-2 compared to TEC KIC-1 and non-TEC controls 46-4 and 73-89. Preparations were viewed by light microscope at 100x objective. Black arrows indicate examples of adherent bacteria.

The adhesion of clinical septicaemic and uroseptic strains was also compared to that of TEC strain HMLN-1 using HT-29 cells only. In all, 5,900 cells infected with the uroseptic strains and 7,798 cells infected with the septicaemic strains were counted for the number of adhering bacteria. It was found that while both the uroseptic and septicaemic strains adhered in a similar manner (i.e. diffuse adhesion), the degree of adhesion among uroseptic strains was $2.90 \pm 3.12$ bacteria/cell (mean ± SD) which
was significantly \( P = 0.0012 \) higher than that of septicaemic strains \( 2.73 \pm 2.92 \) bacteria/cell).

### 2.4.3 Translocation

The ability of the TEC strains investigated in this study to translocate in Caco-2 cells has previously been shown (See Appendix B) (119). However, the route of translocation of these strains has not been documented. In this study, TEC strains were shown to move through Caco-2 cells in a transcellular pathway (Fig. 2.6). Transmission electron microscopy showed that TEC strains adhere tightly to the apical surface of Caco-2 cells (Fig. 2.6A) after their initial adherence to the microvilli (data not shown) and enter cells in a membrane-bound vesicle (Fig. 2.6B).

**Figure 2.6.** Transcellular pathway taken by translocating *E. coli* strain HMLN-1 through gut epithelial Caco-2 cells. (A) Bacteria initially adhered to microvilli at the apical surface, establishing intimate contact with cells, and (B) entered cells in a membrane-bound vesicle. Bacterial cells are indicated by black arrows. Shown are representative images of the transcellular pathway taken by TEC strains HMLN-1, PC-1, KIC-1 and KIC-2. Images shown were photographed by transmission electron microscope at 10 000 and 6 000x magnification, respectively.
The ability of TEC strains to translocate in HT-29 cells was also tested. Experiments were performed once cells had reach stable TEER readings (above 700 Ωcm$^2$) and the results indicated that the overall level of translocation of TEC strains HMLN-1 and PC-1 was significantly ($P < 0.001$) more than rat TEC strains and non-TEC strains used as controls (Fig. 2.7). Rat TEC strains KIC-1 and KIC-2 translocated significantly ($P < 0.05$) more than rat non-TEC controls at 120 min (Fig. 2.7). The TEER, indicating the integrity of the tight junctions, remained constant throughout (Fig. 2.7).

**Figure 2.7.** Rate of translocation of TEC strains in HT-29 cells over 120 min. TEC strain HMLN-1 was the most efficiently translocating one at all time points and, together with PC-1, at 30, 60 and 120 min were translocating significantly more than other strains tested (*$P < 0.001$). Rat TEC strains also translocated significantly more than non-TEC strains at 120 min (#$P < 0.05$). TEER remained stable throughout. Data shown are from triplicate experiments and values are mean ± SEM.
2.4.4 Production of IL-8 in response to TEC strains

The host epithelial immune response to TEC strains was firstly investigated by measuring the kinetics of IL-8 production in HT-29 cells, a cell line often used to test host immune responses (195, 237). TEC strains stimulated a higher level of IL-8 production by HT-29 cells compared to non-TEC strains over 24h (Fig. 2.8). The TEC strain KIC-2 elicited the highest production of IL-8 (Fig. 2.8).

![Figure 2.8. IL-8 production by HT-29 cells during 24 h infection with TEC strains HMLN-1, PC-1, KIC-1 and KIC-2 and non-TEC strains 46-4 and 73-89. KIC-2 elicited the highest proinflammatory response (#P < 0.001) compared to other strains and non-infected cells at 4, 6 and 24 h. When grouped together, TEC strains also elicited a higher IL-8 response than non-TEC strains at 4, 6 and 24 h. Values are mean ± SEM of three replicates.](image)

The kinetics of IL-8 production was also investigated in Caco-2 cells. Preliminary results showed that at early time points, Caco-2 cells did not respond to the same degree as HT-29 cells. Indeed, at 6 h, no difference was observed between infected
cells and non-infected cells. However, testing after 24 h of infection showed differences between TEC and non-TEC strains with highest level of IL-8 produced against TEC strain KIC-2 (Fig. 2.9).

![Bar graph showing IL-8 production by different isolates](image)

**Figure 2.9.** Interleukin-8 production by Caco-2 cells after 24 h infection with TEC and non-TEC strains. TEC strain KIC-2 elicited the highest proinflammatory response compared to all other strains (*P < 0.01). Values are mean ± SEM from three replicates from two independent experiments.

The innate immune response to TEC was thereafter investigated via IL-8 production in monocytic THP-1 cells (Fig. 2.10). While TEC strains collectively elicited a significantly (*P < 0.0001*) higher IL-8 response than non-TEC controls, TEC strain KIC-2 again caused the highest level of IL-8 production compared to all other strains (*P < 0.05*) (Fig. 2.10).
Figure 2.10. Interleukin-8 production by monocytic THP-1 cells after 24 h sensitisation with TEC and TEC strains. Values are mean ± SEM, calculated from three replicates.

2.4.5 IL-8 response to TEC flagellin

In view of high IL-8 production against TEC strain KIC-2 and carriage of the H21 flagellar antigen type which has previously been shown to be immunostimulatory (236), the IL-8 response to monomeric flagellin was investigated to determine if flagellin was indeed responsible for the higher IL-8 production seen against this strain. Flagellin purification and amino acid sequencing revealed 100% protein sequence identity between TEC strains HMLN-1 and PC-1. TEC strain KIC-1 and non-TEC strain 46-4, with identical serotypes, showed 99.8% identity in their flagellin protein sequence. The protein sequence of TEC strain KIC-2 was different to other strains but similar to that of control strain S. typhimurium. Amino acid
sequence alignments of TEC and non-TEC strains are presented in Appendix C. Due to the high similarities in sequences between TEC strains HMLN-1 and PC-1 and between TEC strain KIC-1 and non-TEC strain 46-4, a representative of these strains together with TEC strain KIC-2 were tested for this purpose. The flagellin of TEC strain KIC-2 elicited the highest immune response after 5 h co-incubation with HT-29 cells compared to flagellin from TEC strain PC-1 and non-TEC strain 46-4 (Fig. 2.11).

![Graph showing interleukin-8 production by HT-29 cells in response to 5 h co-incubation with purified monomeric flagellin from representative TEC strains KIC-2, PC-1, and non-TEC 46-4. Flagellin from TEC strain KIC-2 had higher immunostimulatory capacity than that from TEC strain PC-1 and non-TEC 46-4 at all three concentrations tested (*P < 0.05, **P < 0.01 and ***P < 0.001). Values are mean ± SEM, normalised against unstimulated HT-29 cells that were used as controls.]

**Figure 2.11.** Interleukin-8 production by HT-29 cells in response to 5 h co-incubation with purified monomeric flagellin from representative TEC strains KIC-2, and PC-1 and non-TEC 46-4. Flagellin from TEC strain KIC-2 had higher immunostimulatory capacity than that from TEC strain PC-1 and non-TEC 46-4 at all three concentrations tested (*P < 0.05, **P < 0.01 and ***P < 0.001). Values are mean ± SEM, normalised against unstimulated HT-29 cells that were used as controls.
2.5 Discussion

This study showed that TEC strains were able to efficiently adhere to human gut epithelial cells and translocate in a transcellular pathway. It has been shown that not all bacteria are able to move through cells using this pathway but rather, only specific *E. coli* strains, i.e. those capable of translocation, use this route to cross the gut epithelium (142). Overall, TEC strain HMLN-1, of human origin, was the most efficiently adhering and translocating strain followed by pig-origin TEC strain PC-1. While TEC strains originating from rats (KIC-1 and KIC-2) showed a lower efficiency in translocation than HMLN-1 and PC-1, they translocated significantly more than rat non-TEC controls. In general, the rate of adhesion followed a similar trend to that seen in translocation. This suggests that like translocation, adhesion of TEC strains may also be host-species specific and that a higher adhesion rate is associated with a higher translocation rate.

To eliminate the possibility that the TEC interactions observed in this study were not specific to one cell line, adhesion, translocation and IL-8 response to TEC infection were measured in both HT-29 and Caco-2 cells. It is known that differences exist between Caco-2 and HT-29 cells. Caco-2 cells form monolayers, become differentiated over 21 days, form tight junctions between cells and have microvilli similar to mature enterocytes (204). In contrast, HT-29 cells are known to form multiple layers with no tight junction and remain undifferentiated unless this phenotype is induced (45). However, the fact that similar results and/or trends in adhesion, translocation and IL-8 production were observed in both cell lines suggest that these abilities are not dependent on the models used but are rather due to strain-specific virulence properties. Despite this finding, it was concluded that HT-29 cells
may not be the most suitable model for translocation assays. In this cell line, selection of the most suitable time for performing the translocation assay based on TEER values and cell differentiation was difficult to standardise and efforts to identify the route of translocation in this cell line failed due to the lack of tight junctions between these cells. HT-29 cells have previously been used to test bacterial ability to adhere and the host immune response (195, 237). Because data from these experiments were presented in HT-29 cells, the translocation data in this cell line was therefore also presented.

In the present study, it was found that TEC strains HMLN-1 and PC-1 showed high similarity in their adhesion and translocation ability and had identical BPTs and serotype (i.e. O77:H18). This serotype has previously been shown to be associated with extraintestinal pathogenic *E. coli* (109). Furthermore, previous findings by this author have shown that both strains also belonged to phylogenetic group D (187) which generally encompasses strains causing extraintestinal infection (44) and carried the same virulence genes (adhesins *fimH*, and *bmaE* and Group III capsular polysaccharide synthesis gene *kpsMT* III) (187). It has been shown that type 1 fimbriae not only have roles in colonisation and invasion of the urinary tract (66, 161), but are also associated with *E. coli* adhesion to the gut epithelium (90). Since adhesion is the first step towards establishing infection in the host (136), it is possible that the presence of adhesin genes found in TEC strains may mediate their subsequent internalisation and the transcellular passage that was observed in this study. Similar mechanisms may extend to rat TEC strains KIC-1 and KIC-2 which also carried the *fimH* gene. This hypothesis, however, remains to be tested. Finally, since expression of capsule shields bacterial cells against host immune defences (51),
the presence of the kpsMT III gene in TEC strains HMLN-1 and PC-1 may have protective roles and may suggest that survival of these strains in gut-associated lymphoid tissue may be more important than their translocation ability. Nonetheless, the high similarity between TEC strains HMLN-1 and PC-1 indicates that some TEC strains may be pathogens in both human and pigs and probably employ the same mechanisms of action.

Interestingly, in this study, uroseptic strains were more efficiently adhering than septicaemic strains. This was likely due to the higher prevalence of pap genes that these strains carried, as previously shown (Appendix A) (187). P pili, encoded by pap genes, are used by uropathogenic E. coli (UPEC) to adhere and colonise the uroepithelium (238, 242). However, it has been shown that these adhesins may also bind to host cell receptors in the GI tract (81) which may explain the higher adhesion efficiency among uroseptic strains in the present study. Similar to human-origin TEC strain HMLN-1, many of these isolates carried a capsular polysaccharide synthesis gene, with kpsMT II carried by 70% of septicaemic isolates and 83% of uroseptic isolates (Appendix A). This suggests that the bacterial capsule may have similar roles in protecting these strains, as suggested for TEC strain HMLN-1. Unlike strain HMLN-1 which belonged to phylogenetic group D and carried few virulence genes (187), septicaemic and uroseptic strains predominantly belonged to phylogenetic group B2 and carried many virulence genes (see Appendix A). Although lack of virulence genes may indicate less virulence, it is evident that TEC strain HMN-1 can still translocate efficiently which suggests that as yet unknown virulence factors may be involved in this process.
This study also revealed that rat TEC strain KIC-2 adhered almost as efficiently as TEC strains HMLN-1 and PC-1. The virulence gene profiles of these TEC strains have been established previously by this author and it has been shown that rat TEC strains KIC-1 and KIC-2 carried the same adhesins as TEC strains HMLN-1 and PC-1 (187). In comparison to KIC-1 however, KIC-2 additionally carried the EAST1 gene, encoding enteroaggregative heat stable toxin 1 (187). However, the exact role of this toxin gene in this strain remains to be elucidated. While TEC strain KIC-2 belonged to the same phylogenetic group (group B1) as TEC strain KIC-1 and non-TEC strains, it stimulated a much higher IL-8 response. It was hypothesised that this could be attributable to its H21 flagellar antigen which has previously been shown to be immunostimulatory (237). Sequencing showed that the flagellar antigen of TEC strain KIC-2 was highly similar to that of S. typhimurium which has previously been reported to be efficiently recognised by relevant immune components (237). The fact that the purified flagellin of KIC-2 elicited an elevated immune response in comparison to the flagellin of other TEC and non-TEC strains strongly indicates its role in the high IL-8 response seen against this strain. In vivo, the targeting of flagellin by the innate immune system is a host defence against infection (234) and suggests that bacteria carrying immunostimulatory molecules would have reduced chance of survival and therefore reduced opportunity to translocate. On the other hand, TEC strains HMLN-1 and PC-1, although also inducing a higher IL-8 response in comparison to non-TEC strains, may be protected from the host immune response by virtue of their polysaccharide capsule, as described above.

Besides having a role in immune recognition, it has previously been shown that certain flagellar types (e.g. H6 and H7) facilitate colonisation of mucosal surfaces by
attaching/effacing *E. coli* (64). Furthermore, flagella have been shown to be involved in invasion of renal collecting duct cells (182) and brain microvascular endothelial cells (179). Whether the presence of the flagellar types identified here has any role in adhesion, invasion and translocating ability of any of the TEC strains studied is yet to be elucidated. The TEC strain KIC-1 has been shown to be efficient in both adhesion and translocation in its original host (14). In the present study, it was shown to have identical H antigen (H14) type to non-TEC strain 46-4, an adhering but non-translocating strain, and non-TEC strain 73-89, a non-adhering and non-translocating strain (118). In this regard, it can be concluded that the H14 antigen does not have any role in adhesion and/or translocation ability of TEC strain KIC-1.

In conclusion, the results of this study suggest that TEC strains have phenotypic and genotypic characteristics which enable them to efficiently adhere and translocate in a strain-specific transcellular pathway through the gut epithelium. Furthermore, TEC strains may elicit a higher immune response than non-TEC strains. In the case of TEC strain KIC-2, it was shown that its flagellin may contribute to this elevated response. In contrast to previous findings on septicaemic and uroseptic strains, TEC strains carried very few virulence genes. Whether only these or other, as yet unidentified, genes mediated efficient adhesion and translocation among TEC strains remains to be elucidated.

**Next chapter**

This study investigated colonisation and translocation mechanisms of a collection of gut-associated septicaemic *E. coli* strains. Gut *E. coli* are the main source of UTIs and once colonising the urinary tract, may further ascend to the kidneys and into the
blood to cause septicaemia. Colonisation and long-term survival of uropathogenic *E. coli* (UPEC) has been the subject of many studies. However, few have investigated the virulence characteristics which enable *E. coli* to cause UTI in children and pregnant women and whether there is any variation in these characteristics between UPEC isolates from different geographical regions. The next chapter describes an investigation on virulence characteristics that are associated with long-term survival of UPEC in children.
CHAPTER 3
Uropathogenic *E. coli* causing urinary tract infection in children: virulence characteristics associated with long-term survival

3.1 Introduction

Urinary tract infection (UTI) is one of the most common infections in children (35, 144). Prevalence is dependent on factors such as age and gender (213). Males aged <3 months old and females aged <1 year old at highest risk of UTI (213). Overall, UTI occurs in around 2% of male and 8% of female children aged up to 7 years old (89). Of these, 10–30% suffer recurrence (239) which is usually associated with urological abnormalities such as vesicoureteral reflux (VUR) (102, 239). *E. coli* is the most common bacterial species causing UTI in children (33, 35) with a very recent report from the United Kingdom indicating that uropathogenic *E. coli* (UPEC) cause 92% of infections in this patient group (35).

It is recommended that clinicians should carefully monitor and treat children with UTI as infection can cause renal scarring leading to secondary hypertension and chronic kidney disease (101, 130, 144). Children at high risk of recurrent UTI may therefore receive prophylactic treatment to prevent recurrence, renal damage and long-term complications (49, 53, 157). However, recent studies have suggested that prophylactic treatment is ineffective (157) due to increased risk of infection caused by resistant bacteria (49, 53). In the community setting, increased prevalence of resistant organisms causing UTI in children has also been reported (21).
While a number of virulence factors such as adhesins, toxins, siderophores and capsule synthesis components are expressed by UPEC and contribute to its pathogenicity (111), high prevalence of fimH (type 1 fimbriae) and pap (P pili) genes among isolates causing UTI in children (36) indicates the importance of adherence and/or invasion among such isolates. Furthermore, UPEC ability to cause recurrent infection suggests the presence of virulence factors that enable long-term survival in the urinary tract. Investigation of the self-recognising autotransporter protein, antigen (Ag) 43, has shown that this adhesin is involved in UPEC persistence in a mouse model of UTI (230). Additionally, the finding of high prevalence of Ag43 among UPEC isolates causing recurrent UTI in children suggests an association with long-term survival in the human host (137). Investigation of other virulence factors involved in E. coli pathogenesis have shown that curli fimbriae and cellulose, components of biofilms on abiotic surfaces, are also expressed by clinical isolates collected from the urine of patients with community-acquired UTI and, in different ways, promote UPEC survival in the uroepithelium (114).

Despite the high risk of recurrent infection among children, virulence characteristics associated with long-term survival of UPEC are not well studied among isolates from this patient group. Whether differences occur in the prevalence of such virulence factors among isolates from different geographical regions has also not previously been explored.

3.2 Aims of this study

In view of the above, the present study was undertaken to investigate the prevalence of virulence characteristics associated with long-term survival of UPEC in the
urinary tract among isolates causing UTI in young children in 5 different countries.

The first aim of this study was to identify differences in the prevalence of phylogenetic groups among UPEC from the different countries. To investigate whether UPEC causing UTI in this patient group are equipped to survive long-term in the host, the prevalence of Ag43 genes flu, fluA_CFT073 and fluB_CFT073 as well as the incidence of curli and cellulose expression was also investigated. The final aim of this study was to determine the prevalence of antibiotic resistance among isolates investigated and whether associations existed between resistance and other virulence factors which may further promote long-term survival in the urinary tract.

3.3 Materials and methods

3.3.1 Bacterial isolates

A total of 337 UPEC isolates were obtained from urine specimens of children, aged up to 7 years old, who presented with symptomatic UTI in 5 different countries. The samples were collected as clean-catch urine or by suprapubic or urine collecting bag, as appropriate. None of the patients had a catheter or were catheterized during urine sampling. Isolates were collected by clinicians at the Mater Hospital, Brisbane, Australia (n = 80); at Ali-Asghar Children’s Hospital, Tehran, Iran (n = 48); at Comenius University Teaching Hospital and Polyclinic, Bratislava, Slovakia (n = 73); at Astrid Lindgren Children’s Hospital, Karolinska University Hospital, Stockholm, Sweden (n = 98) and at Hanoi University Hospital, Hanoi, Vietnam (n = 38). All isolates from Australia, Slovakia and Sweden were obtained as single-species cultures. Among Iranian and Vietnamese isolates, 46 (96%) and 32 (84%) isolates, respectively, were obtained as single-species cultures. Only one patient, from Sweden, had a concomitant positive blood culture with the same isolate that
was identified in the urine sample. All isolates were received at the laboratory with
general information on how the sampling was performed and the age and gender of
the patients from whom isolates were obtained. However they were blinded to this
author and further access to hospital charts or patient information was not available.
In view of this, and according to the advice received, ethics approval was not
required for this study. *E. coli* identification was confirmed using the Vitek 2 Gram-
negative Identification Card (BioMérieux, Marcy l’Etoile, France) according to the
manufacturer’s instructions.

3.3.2 Extraction of bacterial DNA

Bacterial DNA was extracted using a modified boiling method (106). Briefly,
bacteria were grown overnight on blood agar at 37°C. Colonies were suspended in
sterile microcentrifuge tubes containing sterile deionised water and boiled for 5 min
at 95°C. Samples were then centrifuged at 16 000 x g for 5 min. The supernatant,
containing the DNA, was collected and stored at –20°C.

3.3.3 PCR amplification of virulence genes

The primers used to amplify genes investigated in this study are listed in Table 3.1.
A previously described multiplex polymerase chain reaction (PCR) for the genes
*chuA*, *yjaA* and TSPEC4.C2 was used to phylogenetically group isolates (44). Briefly,
a 20 μl reaction volume consisting of 2 μl of DNA template, 1 μM of each primer,
10x PCR reaction buffer (Invitrogen), 50 mM MgCl₂ (Invitrogen Carlsbad,
California, USA), 1 mM dNTPs, and 1 U of Platinum Taq Polymerase (Invitrogen)
was prepared. The reaction cycle consisted of heating to 94°C for 3 min, 30 cycles of
denaturation (94°C for 5 s) and annealing (59°C for 10 s) and one extension cycle (72°C for 5 min).

**Table 3.1** The primers used to amplify the virulence genes of interest.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Length of PCR product (base pairs)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Multiplex PCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>chuA</em></td>
<td>F: GACGAACCAACGGTCAGGAT &lt;br&gt; R: TGCCGCCAGTACCAAAGACA</td>
<td>279</td>
<td>44</td>
</tr>
<tr>
<td><em>yjaA</em></td>
<td>F: TGAAGTGTCAGGAGACGCTG &lt;br&gt; R: ATGGAGAATGCGTTCCTCAAC</td>
<td>211</td>
<td>44</td>
</tr>
<tr>
<td>TSPE4.C2</td>
<td>F: GAGTAATGTGCGGGCATTCA &lt;br&gt; R: CGCGCCAACGAAATATTACG</td>
<td>152</td>
<td>44</td>
</tr>
<tr>
<td><strong>Uniplex PCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>flu</em></td>
<td>F: GGGTAAAGCTGATAATGTCG &lt;br&gt; R: GTTGCTGACAGTGAGTGTGC</td>
<td>508</td>
<td>230</td>
</tr>
<tr>
<td><em>fluA</em>&lt;sub&gt;CFT073&lt;/sub&gt;</td>
<td>F: AGGCAGGAGGAACTGCCAGT &lt;br&gt; R: TAAATGAGGGTGTCCCGTGCC</td>
<td>340</td>
<td>193</td>
</tr>
<tr>
<td><em>fluB</em>&lt;sub&gt;CFT073&lt;/sub&gt;</td>
<td>F: CAGCCGGATCTGCGGACT &lt;br&gt; R: ACTCTGGTGTTTCTGGCTGT</td>
<td>440</td>
<td>193</td>
</tr>
</tbody>
</table>

All isolates were then tested for the presence of the Ag43 gene, *flu*, as previously described (230). In the PCR, the *flu* gene primers (Table 3.1) amplify copies of all 5 Ag43 allelic variants (193). Isolates found to carry *flu* were further tested for the presence of Ag43 variants, *fluA*<sub>CFT073</sub> and *fluB*<sub>CFT073</sub>, which are both carried by UPEC strain CFT073. The reaction mixtures for amplification of each gene consisted of a total volume of 25 µl containing 1 µl of DNA template, 0.4 µM each of the forward and reverse primer (Table 3.1) and 2x DreamTaq Green PCR Master Mix (Fermentas, Burlington, Canada). The amplification conditions for the *flu* gene consisted of heating to 95°C for 1 min, 30 cycles of 95°C for 30 s, 57°C for 30 s,
72°C for 1 min and one final cycle of 72°C for 5 min. For fluA<sub>CFT073</sub> and fluB<sub>CFT073</sub>, the amplification cycles were as for the flu gene except for the annealing temperatures which were 65°C and 63°C, respectively.

All reactions were performed in a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, Maryland, USA). UPEC strain CFT073 served as a positive control for all genes tested. The reaction product was analysed in a 1.5% agarose gel by electrophoresis and bands were visualised in a GelDoc system (Biorad, Hercules, California, USA) under ultraviolet light following ethidium bromide staining.

3.3.4 Curli and cellulose expression

The expression of curli and cellulose was determined by inspection of the colony morphology of samples grown on Congo red and Calcofluor agar plates at 37°C. Although E. coli may express curli and cellulose at other temperatures, 37°C was selected to mimic the in vivo conditions in the urinary tract. Growth was inspected at 24 and 48 h and scored against positive and negative control strains, as previously described (114). The control strains included wild-type E. coli strain No. 12 expressing both curli and cellulose, knockout strain WE1 (bcsA::Cm) expressing curli only, knockout strain WE11 (csgBA::Cm) expressing cellulose only and knockout strain WE16 (bcsA::Cm csgBA::Cm) lacking both curli and cellulose expression (114).

3.3.5 Antibiotic susceptibility testing

Antibiotic susceptibility testing of all isolates was performed using the Vitek 2 Antimicrobial Susceptibility Tests (ASTN-106) (BioMérieux) according to manufacturer instructions. Susceptibility to ampicillin, amoxicillin/clavulanic acid,
aztreonam, cefalexin, cefotaxime, ceftazidime, ciprofloxacin, ertapenem, gentamicin, mecillinam, nalidixic acid, nitrofurantoin, piperacillin/tazobactam, tobramycin and trimethoprim were tested. Testing enabled identification of extended spectrum beta lactamase (ESBL)-producing isolates. Isolates resistant to antibiotics of 3 or more different classes were classified as multidrug-resistant (MDR) (50).

3.3.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism, Version 5.02 (San Diego, CA, USA). Overall differences between countries regarding the prevalence of virulence genes, factors and antibiotic resistance were tested using the χ² test. Pairwise Fisher’s exact two-tailed tests with correction for multiple comparisons were then performed to identify the country or countries with the most significant differences. \( P < 0.01 \) was considered significant. Fisher’s exact two-tailed test was also used to test for associations between antibiotic resistance and/or virulence factors within individual countries. \( P < 0.05 \) considered significant.

3.4 Results

3.4.1 Patient gender and age

In all, 219 (65%) isolates were obtained from female children and 118 (35%) were from male children. The distribution of male and female patients from each country is listed in Table 3.2. Interestingly, more male children (55%) presented with UTI in Vietnam compared to other countries \( (P < 0.05) \). In all, 50% of the children included in this study were aged one year old or less (Table 3.2).
Table 3.2. Age and gender of children suffering urinary tract infection from whom uropathogenic E. coli isolates were obtained.

<table>
<thead>
<tr>
<th>Country</th>
<th>Gender, n (%)</th>
<th>Age Range</th>
<th>Age (years), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>≤1</td>
</tr>
<tr>
<td>Australia (n = 80)</td>
<td>23 (29)</td>
<td>57 (71)</td>
<td>50 (63)</td>
</tr>
<tr>
<td>Iran (n = 48)</td>
<td>19 (40)</td>
<td>29 (60)</td>
<td>20 (42)</td>
</tr>
<tr>
<td>Slovakia (n = 73)</td>
<td>24 (33)</td>
<td>49 (67)</td>
<td>25 (34)</td>
</tr>
<tr>
<td>Sweden (n = 98)</td>
<td>31 (32)</td>
<td>67 (68)</td>
<td>52 (53)</td>
</tr>
<tr>
<td>Vietnam (n = 38)</td>
<td>21 (55)*</td>
<td>17 (45)</td>
<td>21 (55)</td>
</tr>
<tr>
<td><strong>Total (n = 337)</strong></td>
<td><strong>118 (35)</strong></td>
<td><strong>219 (65)</strong></td>
<td><strong>168 (50)</strong></td>
</tr>
</tbody>
</table>

*More isolates were obtained from male children in Vietnam compared to Australia, Slovakia and Sweden (P < 0.05).

3.4.2 Phylogenetic grouping

Phylogenetic grouping is indicative of the virulence of isolates (44). E. coli belonging to groups B2 and D are generally responsible for extraintestinal infections and carry a higher number of virulence genes than isolates belonging to groups A and B1 (44, 107). Overall, 213 (63%) isolates belonged to phylogenetic group B2, 79 (23%) belonged to group D and 22 (6%) belonged to groups A and B1, respectively (Table 3.3). A number of differences in the distribution of phylogenetic groups were seen among the countries. This included a high proportion (>73%) of isolates from Australia and Slovakia belonging to group B2 while group D was more prevalent among isolates from Iran and Vietnam than among isolates from other countries (Table 3.3). Only one isolate from Vietnam, was not typable using the triplex method as it did not carry any of the 3 genes needed for phylogenetic grouping.
Table 3.3. Phylogenetic grouping of UPEC isolates from children in five different countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Phylogenetic group, ( n (%) )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Australia ((n = 80))</td>
<td>6 (7)</td>
</tr>
<tr>
<td>Iran ((n = 48))</td>
<td>5 (10)</td>
</tr>
<tr>
<td>Slovakia ((n = 73))</td>
<td>2 (3)</td>
</tr>
<tr>
<td>Sweden ((n = 98))</td>
<td>9 (9)</td>
</tr>
<tr>
<td>Vietnam ((n = 38))^c</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

\(^a\)Group B2 isolates from Australia and Slovakia vs. group B2 isolates from Iran \((P < 0.001)\).
\(^b\)Group D isolates from Iran and Vietnam vs. group D isolates from Australia and Slovakia \((P < 0.001)\).
\(^c\)One (2\%) Vietnamese isolate was non-typable.

3.4.3 The prevalence of antigen 43 genes

The high reported incidence of recurrent UTI among children (239) suggests the presence of virulence factors which enable long-term survival of UPEC causing such infections. The prevalence of Ag43 genes, whose expression is associated with UPEC persistence in the uroepithelium (230), was therefore investigated. In all, 242 (72\%) isolates carried the \textit{flu} gene, 116 (48\%) of these carried \textit{fluA}\textsubscript{CFT073} and 110 (45\%) carried \textit{fluB}\textsubscript{CFT073} (Table 3.4). Although the prevalence of the \textit{flu} gene was similar among all countries, differences existed in the prevalence of \textit{fluA}\textsubscript{CFT073} and \textit{fluB}\textsubscript{CFT073} (Table 3.4). These differences included significantly \((P < 0.01)\) lower prevalence of \textit{fluA}\textsubscript{CFT073} among Iranian and Swedish isolates compared to those from other countries while \textit{fluB}\textsubscript{CFT073} was most prevalent among Slovakian isolates (Table 3.4).
Table 3.4. The prevalence of the antigen 43 gene, *flu* and two of its allelic variants (*fluA*$_{CFT073}$ and *fluB*$_{CFT073}$), among uropathogenic *E. coli* from children in the countries investigated.

<table>
<thead>
<tr>
<th>Country</th>
<th>Virulence gene, <em>n</em> (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia ($n = 80$)</td>
<td>54 (68)</td>
<td>32 (40)</td>
<td>26 (33)</td>
</tr>
<tr>
<td>Iran ($n = 48$)</td>
<td>35 (73)</td>
<td>$7 \ (15)^a$</td>
<td>9 (19)</td>
</tr>
<tr>
<td>Slovakia ($n = 73$)</td>
<td>54 (74)</td>
<td>34 (47)</td>
<td>$40 \ (55)^b$</td>
</tr>
<tr>
<td>Sweden ($n = 98$)</td>
<td>68 (69)</td>
<td>$23 \ (23)^c$</td>
<td>24 (24)</td>
</tr>
<tr>
<td>Vietnam ($n = 38$)</td>
<td>31 (82)</td>
<td>20 (53)</td>
<td>11 (29)</td>
</tr>
<tr>
<td><strong>Total ($n = 337$)</strong></td>
<td><strong>242 (72)</strong></td>
<td><strong>116 (34)</strong></td>
<td><strong>110 (33)</strong></td>
</tr>
</tbody>
</table>

$^a$Iran vs. Australia, Slovakia and Vietnam ($P < 0.01$).

$^b$Slovakia vs. Iran and Sweden ($P < 0.0001$).

$^c$Sweden vs. Slovakia and Vietnam ($P < 0.01$).

3.4.4 Expression of curli and cellulose

Overall, curli and cellulose were expressed by 142 (42%) and 54 (16%) isolates, respectively. Differences were observed in the prevalence of curli and cellulose expression among isolates from different countries. No Iranian isolates expressed both curli and cellulose (Fig. 3.1). In turn, the highest prevalence of the curli negative, cellulose negative morphotype was seen among these isolates (Fig. 3.1). The distribution of the curli positive, cellulose positive and curli positive, cellulose negative morphotypes was similar among isolates from Vietnam, Slovakia and Sweden (Fig. 3.1).
Figure 3.1. The prevalence of curli and cellulose expression among uropathogenic *E. coli* from children in five different countries. Abbreviations used: CR: curli, CL: cellulose, +: positive, -: negative. Significant differences: Australia vs. Slovakia (*P* < 0.01); Iran vs. Slovakia, Sweden, Vietnam (**P* < 0.001); Iran vs. all other countries (#P < 0.01).

### 3.4.5 Antibiotic resistance

In general, more isolates from Iran and Vietnam were resistant to antibiotics compared to isolates from other countries (Fig. 3.2). Overall, no isolates were resistant to nitrofurantoin while 2 (4%) isolates, from Iran only, were resistant to ertapenem. Overall, 8 isolates were resistant to mecillinam; however, these were distributed among the 5 countries.
Figure 3.2. The prevalence of antibiotic resistance among 339 uropathogenic *E. coli* isolates from children with urinary tract infection in the countries investigated. Antibiotics shown are those used to treat Gram-negative infection and for which there were significant differences in the prevalence of resistance between countries. Abbreviations used: AM: ampicillin, AMC: amoxicillin/clavulanic acid, ATM: aztreonam, CAZ: ceftazidime, CIP: ciprofloxacin, CN: cefalexin, CTX: cefotaxime, GM: gentamicin, NA: nalidixic acid, TM: tobramycin, TZP: piperacillin/tazobactam, TMP: trimethoprim. Significant differences: Iran and Vietnam vs. all other countries (*P < 0.01, **P < 0.001, ***P < 0.0001); Australia and Iran vs. Slovakia and Sweden; Vietnam vs. Sweden only (#P < 0.01); Iran vs. all other countries (♦P < 0.01).

Following antibiotic susceptibility testing of each isolate, results enabled identification of ESBL-producing and/or MDR isolates (Table 3.5). Prevalence of ESBL-producing and MDR isolates from Iran and Vietnam was significantly (*P < 0.0001) higher than that seen among all other countries (Table 3.5).
Table 3.5. The prevalence of extended spectrum beta lactamase-producing and multidrug-resistant uropathogenic *E. coli* isolates among the countries investigated.

<table>
<thead>
<tr>
<th>Country</th>
<th>ESBL-producing$^a$</th>
<th>MDR$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia ($n = 80$)</td>
<td>2 (3)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>Iran ($n = 48$)</td>
<td><strong>20 (42)$^b$</strong></td>
<td><strong>21 (44)$^b$</strong></td>
</tr>
<tr>
<td>Slovakia ($n = 73$)</td>
<td>1 (1)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Sweden ($n = 98$)</td>
<td>1 (1)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Vietnam ($n = 38$)</td>
<td><strong>15 (39)$^b$</strong></td>
<td><strong>18 (47)$^b$</strong></td>
</tr>
<tr>
<td><strong>Total ($n = 337$)</strong></td>
<td><strong>39 (12)</strong></td>
<td><strong>49 (15)</strong></td>
</tr>
</tbody>
</table>

$^a$Abbreviations used: ESBL: extended spectrum beta lactamase-producing; MDR: multidrug-resistant: isolates resistant to antibiotics belonging to at least 3 different antibiotic classes.

$^b$More Iranian and Vietnamese isolates were ESBL-producing and MDR compared to all other countries ($P < 0.0001$). Data were analysed by $\chi^2$ test and pair-wise Fisher’s exact two-tailed tests. $P < 0.01$ was considered to be significant.

### 3.4.6 *Ag43* associations with phylogenetic groups and resistance

A number of associations were found between the presence of genes *fluA*$_{CFT073}$ and *fluB*$_{CFT073}$ and phylogenetic group B2 (Table 3.6). Common to isolates from all countries except those from Australia, more carrying *fluB*$_{CFT073}$ belonged to group B2 than to other phylogenetic groups (Table 3.6). No associations were found between *Ag43* genes and other phylogenetic groups.
Table 3.6. The distribution of uropathogenic *E. coli* isolates in relation to Ag43 genes and their phylogenetic groups. Only data where significant associations between the presence of Ag43 genes and a phylogenetic group existed are presented.

<table>
<thead>
<tr>
<th>Country and virulence gene</th>
<th>Isolates positive, n (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B2</td>
<td>Non-B2 (A,B1+D)</td>
<td></td>
</tr>
<tr>
<td>Iran (n = 48)</td>
<td>21 (44)</td>
<td>27 (56)</td>
<td>-</td>
</tr>
<tr>
<td><em>fluA</em>&lt;sub&gt;CFI073&lt;/sub&gt; (n = 7)</td>
<td>6 (29)</td>
<td>1 (4)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><em>fluB</em>&lt;sub&gt;CFI073&lt;/sub&gt; (n = 9)</td>
<td>7 (33)</td>
<td>2 (7)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Slovakia (n = 73)</td>
<td>58 (80)</td>
<td>15 (20)</td>
<td>-</td>
</tr>
<tr>
<td><em>fluB</em>&lt;sub&gt;CFI073&lt;/sub&gt; (n = 40)</td>
<td>36 (62)</td>
<td>4 (27)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Sweden (n = 98)</td>
<td>56 (57)</td>
<td>42 (43)</td>
<td>-</td>
</tr>
<tr>
<td><em>fluB</em>&lt;sub&gt;CFI073&lt;/sub&gt; (n = 24)</td>
<td>22 (39)</td>
<td>2 (5)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Vietnam (n = 38)</td>
<td>20 (53)</td>
<td>18 (47)</td>
<td>-</td>
</tr>
<tr>
<td><em>flu</em> (n = 31)</td>
<td>20 (100)</td>
<td>11 (61)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td><em>fluA</em>&lt;sub&gt;CFI073&lt;/sub&gt; (n = 20)</td>
<td>14 (70)</td>
<td>6 (33)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><em>fluB</em>&lt;sub&gt;CFI073&lt;/sub&gt; (n = 11)</td>
<td>11 (55)</td>
<td>0 (0)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

*Data were analysed by pair-wise Fisher’s exact two-tailed tests. *P* < 0.05 was considered to be significant.*

Associations between Ag43 genes and antibiotic resistance were also investigated. Interestingly, 19 (95%) ESBL-producing Iranian isolates carried the *flu* gene compared to only 1 (5%) ESBL-positive isolate that lacked the gene (*P* < 0.01).

Furthermore, associations between resistance to individual antibiotics and the presence of *flu*, *fluA*<sub>CFI073</sub> or *fluB*<sub>CFI073</sub> were found among isolates from Australia, Iran and Sweden (Table 3.7). For instance, among 41 (51%) Australian isolates that were ampicillin resistant, 35 (85%) of these carried the *flu* gene compared to 19 (49%) non-resistant isolates (*P* < 0.001) (Table 3.7).
### Table 3.7. Prevalence of antibiotic resistance among uropathogenic *E. coli* isolates carrying Ag43 genes. Only data where significant associations were found between *flu*, *flu_A*CFT073 or *flu_B*CFT073 and antibiotic resistance are shown.

<table>
<thead>
<tr>
<th>Antibiotic&lt;sup&gt;a&lt;/sup&gt; and virulence gene</th>
<th>Resistance, n (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
<td>Non-resistant</td>
<td></td>
</tr>
<tr>
<td><strong>Australia (n = 80)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>41 (51)</td>
<td>39 (49)</td>
<td></td>
</tr>
<tr>
<td><em>flu</em> (n = 54)</td>
<td>35 (85)</td>
<td>19 (49)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><em>flu_A</em>CFT073 (n = 32)</td>
<td>23 (56)</td>
<td>9 (23)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>AMC</td>
<td>27 (34)</td>
<td>53 (66)</td>
<td></td>
</tr>
<tr>
<td><em>flu</em> (n = 54)</td>
<td>23 (85)</td>
<td>31 (58)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><em>flu_A</em>CFT073 (n = 32)</td>
<td>16 (59)</td>
<td>16 (30)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>NA</td>
<td>8 (10)</td>
<td>72 (90)</td>
<td></td>
</tr>
<tr>
<td><em>flu</em> (n = 54)</td>
<td>8 (100)</td>
<td>46 (64)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><strong>Iran (n = 48)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>37 (77)</td>
<td>11 (23)</td>
<td></td>
</tr>
<tr>
<td><em>flu</em> (n = 35)</td>
<td>30 (81)</td>
<td>5 (45)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>CN &amp; CTX</td>
<td>21 (44)</td>
<td>27 (56)</td>
<td></td>
</tr>
<tr>
<td><em>flu</em> (n = 35)</td>
<td>19 (90)</td>
<td>16 (59)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td><strong>Sweden (n = 98)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMC</td>
<td>9 (9)</td>
<td>89 (91)</td>
<td></td>
</tr>
<tr>
<td><em>flu_B</em>CFT073 (n = 24)</td>
<td>5 (56)</td>
<td>19 (21)</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations used: AM: ampicillin, AMC: amoxicillin/clavulanic acid, CN: cefalexin, CTX: cefotaxime, NA: nalidixic acid.

<sup>b</sup>Data were analysed by pair-wise Fisher’s exact two-tailed tests. *P* < 0.05 was considered to be significant.

A number of associations between phylogenetic group D and antibiotic resistance were also found. Among isolates from Vietnam, 12 (75%) group D isolates were MDR compared to 6 (27%) isolates belonging to other phylogenetic groups (*P* 0.01). Six (38%) Vietnamese group D isolates were resistant to ciprofloxacin compared to
1 (5%) non-group D isolate \((P < 0.05)\). In all, 11 (42%) group D isolates from Sweden were resistant to trimethoprim compared to 11 (15%) non-group D isolates \((P < 0.05)\). No other associations between phylogenetic groups and antibiotic resistance were found.

### 3.4.7 Curli and cellulose and associations with antibiotic resistance

Among Vietnamese isolates only, associations between antibiotic resistance and isolates lacking curli and cellulose were found (Table 3.8a and 3.8b, respectively).

**Table 3.8a.** The prevalence of antibiotic resistance among uropathogenic *E. coli* isolates expressing or lacking curli. Only where significant associations between curli and antibiotic resistance were found are shown.

<table>
<thead>
<tr>
<th>Antibiotic resistancea</th>
<th>Expression, n (%)</th>
<th>(p^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Curli</td>
<td>No curli</td>
</tr>
<tr>
<td>Vietnam ((n = 38))</td>
<td>19 (50)</td>
<td>19 (50)</td>
</tr>
<tr>
<td>ESBL</td>
<td>3 (16)</td>
<td>12 (63)</td>
</tr>
<tr>
<td>MDR</td>
<td>4 (21)</td>
<td>14 (74)</td>
</tr>
<tr>
<td>ATM</td>
<td>3 (16)</td>
<td>16 (68)</td>
</tr>
<tr>
<td>CIP</td>
<td>0 (0)</td>
<td>7 (37)</td>
</tr>
<tr>
<td>CN</td>
<td>5 (26)</td>
<td>13 (68)</td>
</tr>
<tr>
<td>CTX</td>
<td>4 (21)</td>
<td>13 (68)</td>
</tr>
<tr>
<td>NA</td>
<td>4 (21)</td>
<td>13 (68)</td>
</tr>
</tbody>
</table>

\(a\) Abbreviations used: ESBL: extended spectrum beta lactamase-producing, MDR: multidrug-resistant, ATM: aztreonam, CIP: ciprofloxacin, CN: cefalexin, CTX: cefotaxime, NA: nalidixic acid.

\(b\) Data were analysed by pair-wise Fisher’s exact two-tailed tests. \(P < 0.05\) was considered to be significant.
Table 3.8b. The prevalence of antibiotic resistance among uropathogenic *E. coli* isolates expressing or lacking cellulose. Only where significant associations between cellulose and antibiotic resistance were found are shown.

<table>
<thead>
<tr>
<th>Antibiotic resistance&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Expression, n (%)</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cellulose</td>
<td>No cellulose</td>
</tr>
<tr>
<td>Vietnam (n = 38)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESBL</td>
<td>0 (0)</td>
<td>15 (50)</td>
</tr>
<tr>
<td>ATM</td>
<td>0 (0)</td>
<td>16 (53)</td>
</tr>
<tr>
<td>TMP</td>
<td>4 (50)</td>
<td>26 (87)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations used: ESBL: extended spectrum beta lactamase-producing, ATM: aztreonam, TMP: trimethoprim.

<sup>b</sup>Data were analysed by pair-wise Fisher’s exact two-tailed tests. *P* < 0.05 was considered to be significant.

### 3.4.8 Associations between age, gender and virulence characteristics.

In a number of instances, patient gender was associated with phylogenetic group B2. Among Slovak isolates, 23 (96%) from male children belonged to group B2 compared to 35 (74%) isolates originating from female children (*P* < 0.05). In addition, 23 (71%) isolates from Swedish male children belonged to group B2 compared to 33 (49%) isolates from female children (*P* < 0.05). Among isolates from Vietnam, 15 (71%) from male children carried the *fluACFT073* gene compared to 5 (29%) isolates from females (*P* < 0.05). No associations were found between gender and antibiotic resistance or with the expression of curli and cellulose. Furthermore, the age of patients was not significantly associated with any of the virulence properties tested.

### 3.5 Discussion

In this study, 50% of all isolates originated from children aged less than 1 year old which is in line with previous reports of high prevalence of UTI among this age
group compared to children of other ages (213). While there was a high prevalence of Ag43 genes among isolates from most countries, fewer Iranian isolates carried these. Expression of curli and cellulose was also less common among Iranian isolates. However, prevalence of antibiotic resistance was highest among these and Vietnamese isolates. The presence of Ag43 genes was associated with antibiotic resistance among isolates from Australia, Iran and Sweden, suggesting that these isolates may have enhanced ability to survive in the urinary tract. On the other hand, among Vietnamese isolates, resistance was associated with the lack of expression of curli and cellulose.

Isolates from Australia, Slovakia and Sweden predominantly belonged to phylogenetic group B2, followed by group D and to a lesser extend groups A and B1. This trend is similar to that seen among intestinal isolates from the faeces of Swedish infants (173) and among isolates causing community-acquired (29, 50) and nosocomial bacteraemia (50) in Denmark and Ireland, respectively. Interestingly, high prevalence of phylogenetic group D was observed among isolates from Iran and Vietnam. Group D strains are associated with extraintestinal infection (44) and may carry similar and/or different virulence genes to group B2 strains (107) indicating that UPEC causing UTI in children in Iran and Vietnam may have different virulence profiles to isolates causing infection in other countries.

The flu gene was found to be highly prevalent (among 72% of isolates) in this study suggesting that Ag43 may play a dominant role in the survival of these isolates in the urinary tract. It is noteworthy that the prevalence of the flu gene may be similar among isolates causing UTI and those causing asymptomatic bacteriuria in adults.
However, the presence of other virulence genes, including those encoding adhesins, likely enhance UPEC ability to cause cystitis and pyelonephritis compared to strains causing asymptomatic infection (139). Although the presence of other virulence genes such \( \text{fimH} \) and the \( \text{pap} \) genes were not investigated in the present study, their high prevalence among isolates causing UTI in children have previously been demonstrated (36). Furthermore, the overall prevalence of \( \text{fluA}_{\text{CFT073}} \) and \( \text{fluB}_{\text{CFT073}} \) (34\% and 33\%, respectively) was higher than that previously reported among UTI and commensal \( E. \ coli \) strains. Restieri et al. (2007) (193) reported that 29\% and 19\% of UTI isolates carried \( \text{fluA}_{\text{CFT073}} \) and \( \text{fluB}_{\text{CFT073}} \), respectively, while 15\% and 10\% of commensal isolates from the \( E. \ coli \) Reference Collection, carried \( \text{fluA}_{\text{CFT073}} \) and \( \text{fluB}_{\text{CFT073}} \), respectively. Differences in the prevalence of these genes were also noted among isolates from different countries, including higher prevalence among isolates from Australia, Slovakia and Vietnam compared to those from Iran and Sweden. It remains to be elucidated whether similar variations exist in the prevalence of other UPEC-associated virulence factors such as the autotransporter protein \( \text{UpaH} \) which has recently been shown to have functions similar to Ag43 (6).

Although 5 Ag43 allelic variants have been described, only UPEC CFT073-associated variants \( \text{fluA}_{\text{CFT073}} \) and \( \text{fluB}_{\text{CFT073}} \) were investigated in this study in view of the functional roles that these variants have been shown to have in UPEC persistence in the urinary tract (230) and because of their association with UTI isolates (193). Although little is known about the roles of other variants, all have been shown to be involved in biofilm-like formation, which may also facilitate their persistence in the host (7, 123). However, their roles in the pathogenesis of UPEC are yet to be fully elucidated. Among Iranian isolates, the \( \text{flu} \) gene was equally prevalent compared to
other countries however, the prevalence of \(fluA_{\text{CFT073}}\) and \(fluB_{\text{CFT073}}\) was found to be significantly lower. Since \(E.\ coli\) may carry multiple allelic variants (194), it is probable that isolates from Iran may predominantly carry other variants than those investigated which may nevertheless facilitate their survival in the urinary tract.

In this study, isolates carrying Ag43 genes predominantly belonged to phylogenetic group B2 and were resistant to a number of beta lactam antibiotics (ampicillin, amoxicillin/clavulanic acid, cefalexin and cefatoxime). In contrast, \(fluA_{\text{CFT073}}\) has previously been associated with phylogenetic group D and with fluoroquinolone resistance (138). In all, the differences between the isolates tested here and previous findings on Ag43 were not unexpected due to the varying mechanisms and virulence characteristics that are associated with UPEC causing urosepsis compared to those causing UTI (36).

Recent findings have shown that curli and cellulose expression is prevalent among UPEC isolates collected from the fresh urine of patients with community-acquired UTI (114). While curli facilitate adherence and protect UPEC against the human antimicrobial peptide LL-37, they also trigger a proinflammatory response (114). On the other hand, cellulose protects UPEC by reducing the stimulation of the immune system (114). Although the expression of curli has been linked with resistance to antibacterial agents such as chlorine (200) and hydrogen peroxide (228), no studies have investigated whether these adhesins may be associated with antibiotic resistance. In the present study, antibiotic resistance was associated with the lack of expression of curli and cellulose among UPEC isolates from Vietnam. This suggests that curli and cellulose play subordinate roles in antibiotic resistance and long-term
survival of bacteria causing UTI in children in this country. Furthermore, it is known that bacteria may develop antibiotic resistance at the expense of expression of other virulence factors (8). It is therefore likely that despite reduced expression of factors that could facilitate colonisation, the high level of antibiotic resistance among UPEC causing UTI in Vietnamese children may nevertheless enable their long-term survival in the host.

The present findings on the prevalence of resistance in each of the countries investigated likely reflect differences in availability and consumption/use of antibiotics. Over the last 10–20 years, surveillance programs aimed at decreasing rising levels of resistance have shown reductions in the prescription and use of antibiotics in Australia (177), Slovakia (232) and Sweden (155). In contrast, in Iran and Vietnam antibiotics can often be bought over-the-counter, leading to increased risk of inappropriate use in the community and rising levels of bacterial resistance (61, 152). Reflecting this, a high prevalence of ESBL production (>39%) and multidrug-resistance (>42%) was found among Iranian and Vietnamese isolates. On the other hand, lower prevalence (<6%) among isolates from Australia, Slovakia and Sweden was similar to levels reported among uropathogens causing infection in children in the USA (21, 78). Treatment of UTI caused by ESBL-producing and MDR isolates may be challenging due to the reduced number of antibiotic options available (21, 78) and may be associated with increased risk of renal scarring in children (164). Taken together, results from this study suggest that children in Iran and Vietnam are at higher risk of recurrent infection and possible renal damage due to the high prevalence of resistance, compared to children in countries such as Australia, Slovakia and Sweden.
Among Vietnamese isolates, phylogenetic group D was highly prevalent and was associated with multidrug-resistance. Such association has previously been described in *E. coli* causing bacteraemia (29, 50, 104) and UTIs in adults (108). It is suggested that bacterial and environmental determinants may both enable increased development of resistance among non-B2 groups (108). Since non-B2 groups were predominant among UPEC from Iran and Vietnam, and in view of antibiotic use practices which promote the development of resistance in these countries, it is perhaps therefore not surprising that a high prevalence of resistance was observed among these isolates.

In conclusion, this study highlights the role of Ag43 in the pathogenesis of UPEC in young children in the countries investigated. In addition, this study shows significantly high prevalence of antibiotic resistance among isolates from Iran and Vietnam, including resistance to multiple classes of antibiotics. Based on these findings, curli and cellulose do not seem to play key roles in antibiotic resistance of UPEC isolates and may rather be factors whose expression is sacrificed in the development of antibiotic resistance. This study underscores the challenges facing clinicians in treating children with UTI caused by highly resistant UPEC while addressing the problem that these bacteria can also carry virulence factors that further facilitate their long-term survival in children.

In the next chapter of this thesis, the virulence genes and factors tested here, together with prevalence of antibiotic resistance were investigated among isolates causing
UTI in pregnant women, another patient group at high risk of UTI and recurrent infection.
CHAPTER 4

Virulence characteristics associated with long-term survival of uropathogenic *E. coli* in pregnant women

4.1 Introduction

Urinary tract infection is the predominant type of bacterial infection among pregnant women (171, 176, 209). *E. coli* is the primary causative bacterial species, causing as many as 90% of infections (57, 171, 176, 209). Other bacterial species that may cause UTI include *Proteus mirabilis*, *Klebsiella pneumoniae* and Gram-positive species such as Group B streptococcus and *Staphylococcus saprophyticus*, to a lesser degree (57, 209). Overall, UTI may be suffered in up to 20% of pregnancies (149). Symptomatic infections such as acute cystitis or pyelonephritis occur in up to 4% and 2% of pregnant women, respectively (149, 171) with pyelonephritis occurring commonly during the second trimester (214). Interestingly, incidence of UTI in the non-pregnant condition significantly elevates the risk of infection during pregnancy (171). Additionally, there is high risk of recurrent infection among pregnant women although the mechanisms underlying this are not fully elucidated (171).

Asymptomatic bacteriuria may be suffered by around 10% of pregnant women (171). Increased risk of development of acute pyelonephritis and adverse effects on the foetus necessitates that pregnant women are screened and treated with antibiotic therapy if they are found to be suffering from this condition (46, 151, 169). Although Group B streptococcus infection accounts for just 1% of cases of asymptomatic bacteriuria (151), its association with preterm delivery, early onset neonatal sepsis


and mortality indicates the importance of treatment to prevent such negative outcomes (151, 209).

A number of factors are associated with high incidence of UTI among pregnant women. Among these, changes in maternal steroid hormones may facilitate UTI by increasing expression of decay accelerating factor which functions as a receptor for uropathogens (171). Physical changes in the urinary tract, including ureteral dilatation and changes in bladder volume and tone, also promote UTI (62, 171, 214). Such changes are likely to be responsible for the high incidence of acute pyelonephritis in the second trimester (214).

UTI during pregnancy can be dangerous for both the mother and foetus. Sufferers may experience symptoms such as fever, flank pain, nausea and vomiting (171). Additionally, hospital-based medical treatment and parenteral antibiotic therapy may be required to restore hydration and fight infection (171). In this respect, treatment can be more challenging if the causative agent is resistant to antibiotics (176). Common complications associated with UTI during pregnancy include pre-term delivery and increased incidence of intra-uterine growth restriction (149). To a lesser degree, pre-eclampsia, caesarean delivery, anaemia, sepsis and septic shock may also be associated with UTI in these patients (149). It is noteworthy that among women who have suffered acute pyelonephritis, the development of renal scars is more common among pregnant patients in comparison to non-pregnant women (189). Overall, the importance of understanding the virulence characteristics of UPEC causing UTI in pregnant women is underscored by the seriousness of the
complications that may be suffered by both the mother and foetus following maternal infection.

The possession of virulence factors which enable colonisation is important in the pathogenesis of UPEC causing infection in pregnant women. It has been shown that more than 50% of UPEC strains causing acute pyelonephritis in these patients express P pili (172). However, expression is most prevalent among isolates causing infection in the second trimester (172). In contrast, type 1 fimbriae are expressed predominantly in the first trimester with prevalence decreasing among isolates causing infection in later stages of pregnancy (172). Dr fimbriae are also expressed by pyelonephritic strains, although to a lesser degree than both P pili and type 1 fimbriae (172). Interestingly, a number of virulence genes including hly (encoding haemolysin), iha (encoding adhesin), papC (encoding P pili) and iroN (encoding siderophore) have been found to be more prevalent among isolates colonising the vagina of pregnant women compared to non-pregnant women (84). Such findings suggest that UPEC strains colonising this site prior to UTI may be more virulent than those infecting other women (84). As with children, the prevalence of virulence factors associated with long-term survival of UPEC in pregnant women are not well understood.

4.2 Aims of this study

In view of the high prevalence of UTI caused by UPEC in pregnant women and the risks associated with recurrent infection, this study was undertaken to investigate the prevalence of virulence factors associated with long-term survival in the urinary tract including antigen 43, curli and cellulose among isolates from these patients. To gain
a better understanding of the variations in characteristics of UPEC in different geographical regions, isolates from Sweden and Uganda were compared. Differences in phylogenetic grouping and the prevalence of antibiotic resistance among UPEC isolates from these countries were also investigated.

4.3 Materials and methods

4.3.1 Bacterial isolates

Between October 2009 and May 2010, a total of 88 UPEC isolates were obtained as pure culture from urine specimens of pregnant women attending outpatient clinics in Stockholm, Sweden (n = 50) and at Mulago Hospital, Kampala, Uganda (n = 38). All isolates were confirmed to be E. coli using the Vitek 2 Gram-negative Identification Card (BioMérieux, Marcy l’Etoile, France) according to manufacturer instructions.

4.3.2 Virulence genes

The extraction of bacterial DNA and PCR amplification of virulence genes chuA, yjaA, TSPEC4.C2, flu, fluA_CFT073 and fluB_CFT073 was performed as described in Chapter 3, Sections 3.3.2 and 3.3.3.

4.3.3 Curli and cellulose

The expression of curli and cellulose was tested following overnight growth of isolates on Congo red and Calcofluor agar plates as described in Chapter 3, Section 3.3.4.
4.3.4 Antibiotic resistance

All isolates were tested for antibiotic susceptibility using Antimicrobial Susceptibility Tests (ASTN-106) (BioMérieux) as described in Chapter 3, Section 3.3.5.

4.3.5 Statistical analysis

Fisher’s exact two-tailed test was used to test prevalence of virulence factors and antibiotic resistance between isolates from the two different countries. \( P < 0.05 \) considered significant.

4.4 Results

4.4.1 Phylogenetic grouping

Phylogenetic grouping revealed that 52% of the Swedish isolates belonged to phylogenetic group B2 which was significantly \( (P < 0.05) \) more than 29% of isolates from Uganda. In contrast, among Ugandan isolates, more belonged to phylogenetic group B1 (32%) compared to isolates from Sweden (6%) \( (P = 0.0003) \) (Table 4.1).

Four (8%) isolates from Swedish pregnant women did not carry any of the three virulence genes that are used to determine phylogenetic grouping and were therefore not able to be grouped using the method employed.
Table 4.1. Phylogenetic group distribution among uropathogenic *E. coli* isolates from pregnant women in Sweden and Uganda.

<table>
<thead>
<tr>
<th>Country</th>
<th>Phylogenetic group, n (%)</th>
<th>A</th>
<th>B1</th>
<th>B2</th>
<th>D</th>
<th>NT^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweden (n = 50)</td>
<td></td>
<td>7 (14)</td>
<td>3 (6)</td>
<td>26 (52)^b</td>
<td>10 (20)</td>
<td>4 (8)</td>
</tr>
<tr>
<td>Uganda (n = 38)</td>
<td></td>
<td>10 (26)</td>
<td>12 (32)^c</td>
<td>11 (29)</td>
<td>5 (13)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

^aNT: non-typable.
^bGroup B2: Sweden vs. Uganda (P = 0.0491).
^cGroup B1: Uganda vs. Sweden (P = 0.003).

4.4.2 Prevalence of antigen 43 genes

Overall, the *flu* gene was found to be carried by 58% of isolates, while *fluA* and *fluB* were found among less than 20% of isolates (Table 4.2). Although no differences were found between the two countries in the prevalence of *flu* and *fluA*, more Swedish isolates carried *fluB* compared to those from Uganda (Table 4.2).

Table 4.2. The prevalence of the antigen 43 genes, *flu*, *fluA* and *fluB*, among uropathogenic *Escherichia coli* from pregnant women in Sweden and Uganda.

<table>
<thead>
<tr>
<th>Country</th>
<th>Virulence gene, n (%)</th>
<th>flu</th>
<th><em>fluA</em></th>
<th><em>fluB</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweden (n = 50)</td>
<td></td>
<td>31 (62)</td>
<td>5 (10)</td>
<td>10 (20)^a</td>
</tr>
<tr>
<td>Uganda (n = 38)</td>
<td></td>
<td>20 (53)</td>
<td>9 (24)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Total (n = 88)</td>
<td></td>
<td>51 (58)</td>
<td>14 (16)</td>
<td>11 (13)</td>
</tr>
</tbody>
</table>

^a*fluB*: Sweden vs. Uganda (P = 0.02).

4.4.3 Expression of curli and cellulose

The expression of curli and cellulose was not prevalent among UPEC isolates from pregnant women. In all, 32 (36%) isolates expressed curli while 14 (16%) expressed
The prevalence of the different curli and cellulose morphotypes was similar between isolates from Sweden and Uganda (Fig 4.1).

**Figure 4.1.** The prevalence of combined curli and cellulose expression among uropathogenic *E. coli* in pregnant women in Sweden (*n* = 50) and Uganda (*n* = 38). Abbreviations used: CR: curli, CL: cellulose, +: positive, -: negative.

### 4.4.4 Antibiotic resistance

A high prevalence of resistance to ampicillin, amoxicillin/clavulanic acid, cefalexin, trimethoprim and piperacillin/tazobactam was found among Ugandan isolates which was significantly (*P* < 0.05) more that found among Swedish isolates (Fig 4.2). Resistance to a number of antibiotics, including aztreonam, ceftazidime, ciprofloxacin, cefotaxime, nitrofurantoin, gentamicin, mecillinam and tobramycin, occurred in less than 10% of all isolates while no isolate was resistant to ertapenem (Fig. 4.2). In all, no associations were found between antibiotic resistance, phylogenetic groups, the presence of Ag43 genes or the expression of curli and cellulose.
Figure 4.2. The prevalence of antibiotic resistance among uropathogenic *E. coli* isolates causing urinary tract infection in pregnant women in Sweden and Uganda. Abbreviations used: AM: ampicillin, AMC: amoxicillin/clavulanic acid, ATM: aztreonam, CAZ: ceftazidime, CIP: ciprofloxacin, CN: cefalexin, CTX: cefotaxime, ETP: ertapenem, FT: nitrofurantoin, GM: gentamicin, MEC: mecillinam, NA: nalidixic acid, TM: tobramycin, TMP: trimethoprim, TZP: piperacillin/tazobactam. Significant differences between the two countries (#*P* < 0.05, *P* < 0.01, ***P* < 0.001).

### 4.5 Discussion

Findings from this study showed differences in phylogenetic groups between isolates causing UTI in pregnant women in Sweden compared to in Uganda. The predominance of phylogenetic group B2 among Swedish isolates generally indicates that such isolates have enhanced ability to cause UTI compared to those belonging to other phylogenetic groups. Furthermore, the distribution of groups is similar to that previously reported in other patient groups, including adults suffering bacteraemia (29, 50). In contrast however, isolates from pregnant women in Uganda were more
equally distributed among phylogenetic group A, B1 and B2. Since groups A and B1 generally represent commensal isolates (44) carrying fewer virulence genes than group B2 and D isolates (107), these findings indicate that more UTIs in Ugandan patients were caused by less virulent UPEC than among Swedish pregnant women. Since disease caused by commensal strains is usually associated with immunocompromised patients (3, 115), it could be speculated that these findings indicate reduced immunity among Ugandan pregnant women in comparison to Swedish patients; however, this hypothesis remains to be explored. Alternatively, it is possible that Ugandan UPEC isolates belonging to groups A and B1 may carry specific virulence factors enabling colonisation of the urinary tract which are not commonly found among these phylogenetic groups elsewhere.

In this study, the overall prevalence of Ag43 gene *flu* and its allelic variants was low. Since Ag43 is involved in long-term survival of UPEC in the urinary tract (230), this finding may suggest that isolates from pregnant women are less equipped to persist in the host than isolates carrying Ag43 and/or may rely on other virulence factors for this. While similar prevalence of the genes *flu* and *fluA* 

CFT073 was observed among the two countries, *fluB* 

CFT073 was more prevalent among isolates from Sweden. Overexpression of *fluB* 

CFT073 has been shown to reduce the level of colonisation during early stages of infection (230), therefore, the overall low prevalence (<15%) of this gene among isolates from Uganda suggests that *fluB* 

CFT073 does not play a major role in UPEC pathogenesis in this patient cohort. Moreover, lower prevalence of Ag43 in comparison to UPEC isolates from children (see Chapter 3, Table 3.4) further supports this indication. Like Ag43, the low prevalence of curli and cellulose expression among isolates from pregnant women suggested that these are also not
key factors in the pathogenesis of these bacteria. As discussed in Chapter 3, Section 3.5, it is likely that curli and cellulose are virulence factors that are lost as UPEC develop antibiotic resistance.

Treatment of UTI in pregnant women is highly important to prevent complications that can endanger both the mother and foetus (39, 171). In treatment itself, however, careful consideration must be taken of the antibiotics used as some may have adverse effects, including possible teratogenic activity (39). Clinically, β lactams, cephalosporins and nitrofurantoin are the first-line antibiotics chosen to treat pregnant women (39, 128). In the present study, significantly more Ugandan isolates were resistant to β lactam antibiotics such as ampicillin and amoxicillin and the cephalosporin, cefalexin, compared to Swedish isolates. The findings of this study suggest that Ugandan clinicians face a greater challenge in treating UTI in pregnant women in comparison to those in Sweden and indicate the need for additional antibiotic options. Here, nitrofurantoin may serve as a suitable alternative first-line treatment option (39, 128) as resistance was very low among all isolates. Overall, the highest incidence of resistance among Ugandan isolates was seen against trimethoprim which was significantly more than among Swedish isolates. While trimethoprim is not a suitable treatment option in the first trimester of pregnancy due to possible adverse effects on neural tube development (39, 128), it may be used to treat UTI in later stages of pregnancy (39). However, the level of resistance seen here would indicate that trimethoprim could not be considered a primary treatment option among Ugandan pregnant women. The levels of resistance seen in this study likely reflect the varying practices in antibiotic use in different countries. Low prevalence of resistance seen among
Swedish isolates may be associated with a national program which is in place to reduce the use of antibiotics and development of resistance (155). Interestingly, the prevalence of resistance seen among Swedish isolates was similar to that reported among *E. coli* causing UTI in pregnant women in Tanzania (24). Although antibiotics can be bought in grocery stores in Tanzania, Blomberg *et al.* (2005) (24) hypothesised that few people in the rural study area would have had access to such stores which may have accounted for the low rates of resistance that were observed among those isolates. On the other hand, in rural areas of Uganda, antibiotics are available through private providers and self-medication, which promotes the development of resistance, is common (125). It may be expected that in a city like Kampala, where isolates were obtained, antibiotics may be equally or more readily available than in rural areas.

Overall, findings of this study suggested that judging on the basis of phylogenetic grouping and the low level of virulence factors that promote long-term survival, UPEC strains isolated from pregnant women are not highly virulent. Whilst it seems likely that anatomical and hormonal changes during pregnancy enable such isolates to cause UTI in these patients, it is also possible that this could be partly due to a lower host immune defence in Ugandan women which enabled strains with lower virulence to still colonise the host and cause infection. Alternatively, Ugandan isolates might have virulence properties that specifically evolved to allow their colonisation of pregnant women in this country.

Findings presented in this and the previous chapter show high prevalence of resistance to commonly used antibiotics among UPEC causing UTI in Ugandan
pregnant women and Iranian and Vietnamese children. In many countries there is a growing interest in treatment using traditional medicine. One such traditional medicine is the herb, *Labisia pumila var. alata* which is commonly used by Malaysian women to treat many ailments. In view of rising levels of antibiotic resistance and the public interest in traditional medicine to treat UTI, the following chapter investigates the effect of the aqueous extract of this herb on the colonisation of UPEC in a uroepithelial cell line.
CHAPTER 5

The protective efficacy of *Labisia pumila* var. *alata* aqueous extract on uropathogenic *E. coli* infection in a cell culture model of the human uroepithelium

5.1 Introduction

Urinary tract infection (UTI) is a highly prevalent bacterial infection which is associated with significant medical and economic burden (75, 93). Many different patient groups may suffer UTI, however, prevalence and risk of recurrence is highest among certain patient groups, including pregnant and postmenopausal women (75, 92). Although different bacterial species may be capable of causing UTI (196), uropathogenic *E. coli* (UPEC) are the most commonly implicated bacteria causing this infection (54, 115, 196, 198).

In the uroepithelium, UPEC adhesion to bladder epithelial cells initiates colonisation (160). Adhesion is mediated by both bacterial factors, such as type 1 fimbriae and P pili (161), and by host receptors (54). Following adhesion, bacterial invasion of superficial bladder epithelial cells (163) and the formation of intracellular bacterial communities enables UPEC persistence in the urinary tract (7, 19, 161, 162). However, despite aiding colonisation, bacterial adhesion and invasion may also trigger the protective host immune response (161, 162). Indeed, upon stimulation, the host may employ a number of mechanisms, including apoptosis and exfoliation of infected cells, as part of the innate defence against UTI (161).
High worldwide incidence of UTI (198), exacerbated by increasing number of infections caused by the emergence and spread of multi-drug resistant UPEC strains (86) presents significant challenges in treatment. Scientists therefore started investigating the possible efficacy of medicinal plant products to prevent and/or treat UTI (83, 129). Studies in this area have identified anthocyanins, antioxidant components of fruits (83, 129, 249) and other plants (215), to induce apoptosis in human hepatoma (215) and rat oesophageal epithelial cells (249). Interestingly, anthocyanin has recently been identified in the aqueous extract of Labisia pumila var. alata (LPva) (170), a well-known traditional medicinal herb found naturally or cultivated in Malaysia (245).

LPva, also known as Kacip Fatimah and belonging to the Myrsinaceae plant family (170), is usually boiled in water and the broth taken as a drink (31). It is traditionally used to treat a number of female-related health conditions including menstrual irregularity and pain, and as post-partum medication (31). Such uses indicate that LPva may have oestrogen-like properties. In addition, the phytoestrogenic activities of LPva have been shown in ovariectomised rats (72). Although the roles of oestrogen in UTI are not well understood, postmenopausal women typically have lower levels than other females (62) and are at higher risk of UTI (95). In view of such observations, clinicians have explored the effects of oestrogen therapy and have shown that among postmenopausal patients, treatment decreased the incidence of recurrent infection (65, 181, 188, 190). Collectively, the antioxidant and the oestrogen-like properties of LPva, suggest that this traditional medicinal herb may have potential applications against bacterial infection of the uroepithelium.
5.2 Aims of this study

In view of the above, the aims of this study were to investigate the efficacy of the aqueous extract of LPva for prevention and/or treatment of UPEC infection in a cell culture model of the uroepithelium and to determine the underlying mechanisms of this action. Since apoptosis is a protective innate defence mechanism in the urinary tract, the first aim of this study was to investigate whether LPva induced apoptosis in bladder epithelial cells. The second aim of this study was to examine the effects of LPva extract on the expression of proteins including apoptosis-promoting caveolin-1 and invasion-mediating β1 integrin. The third aim of this study was to elucidate whether LPva exhibited antimicrobial properties or elicited antimicrobial responses in bladder epithelial cells. The final aim of this study was to investigate whether LPva extract had any effect on the level of bacterial adherence and/or invasion of uroepithelial cells.

5.3 Materials and methods

5.3.1 Cell culture and bacteria

The human uroepithelium was modelled using the T24 human bladder epithelial cell line (ATCC HTB-4). T24 cells (passages 47–57) were maintained in McCoy’s 5A medium with L-glutamine (Gibco, Carlsbad, CA, USA) and 10% foetal bovine serum (Gibco). At confluence, cells were detached from flasks using 0.05% trypsin 0.03% EDTA in PBS. Cells were resuspended in 13 ml of fresh medium and then diluted again 1:6. The diluted cell suspension was then aliquoted into 6- or 24-well tissue culture plates (Sarstedt Inc, Newton, NC, USA and Costar, Corning, NY, USA) in volumes of 2 ml or 1 ml, respectively. During routine culture and the infection
periods of experiments, cells were maintained at 37°C in a humidified incubator with 5% CO₂.

A well-established UPEC strain, CFT073, was used to infect T24 cells. A loopful of culture was grown overnight on blood agar at 37°C. Thereafter, a single colony was taken and suspended in Luria-Bertani (LB) broth. Bacteria were then cultured for 3–4 h to logarithmic phase on a reciprocal shaker (100 strokes per min, 37°C). The bacterial culture was then centrifuged (2500 x g, 10 min), the supernatant discarded, and the pellet resuspended in phosphate-buffered saline (PBS). The concentration of bacterial suspension was determined by spectrophotometer at 600 nm. A final concentration of approximately 1.0 x 10⁷ CFU/ml was used for infections.

5.3.2 LPva treatment

A standardised LPva aqueous extract, kindly provided by Professor MM Yusoff from University Malaysia Pahang, was prepared as previously described (72). Briefly, LPva plant material was washed and then dried over 3 days at 40°C. Thereafter, the dried material was placed in distilled water (1:6) and was heated at 80°C for 3 h, with constant stirring to enable extraction. The resulting liquid was filtered and then spray-dried to acquire the LPva extract. A stock solution was prepared by resuspending the dried extract in sterile deionised water and was filter sterilised using a 0.45 µm syringe filter. For treatment of cells, the stock solution was diluted in cell culture medium to final concentrations of 10 µg/ml, 100 µg/ml and 1000 µg/ml.
T24 cells, grown overnight in 6- or 24-well plates were treated with different concentrations of LPva extract. In general, the concentration used for infections was 100 μg/ml. This concentration was determined by extrapolation of doses used in previous animal experiments (72). For all experiments, medium containing LPva was renewed daily and experiments were performed after 6, 24 or 72 h of treatment. Non-treated (control) cells were grown and handled in parallel with LPva-treated cells. Preliminary testing showed few cells remained attached to wells following 72 h treatment with 1000 μg/ml LPva. This concentration was therefore only used where the purpose was to test the effect of a high concentration of LPva on apoptosis.

Viability and proliferation of T24 cells treated for 72 h with LPva extract (100 μg/ml) was confirmed in comparison to non-treated cells using the trypan blue dye exclusion test and the XTT assay. Briefly, culture medium was removed from T24 cells grown in 24-well plates and 0.4% trypan blue (Sigma-Aldrich, St Louis, MO, USA) was added for 5 min. Cells were then washed three times with PBS and assessed by light microscope. Those staining blue were deemed non-viable (222). The XTT assay was performed in a modified method to that previously described (2, 210). Briefly, the cell culture medium was removed from cells and wells were washed with PBS. Cells were then incubated for 4 h at 37°C with XTT solution (Sigma-Aldrich) and 12.5 μM menadione. The reaction solution was read at 490 nm to confirm cell viability and proliferation of LPva-treated cells compared to non-treated control cells.

### 5.3.3 Apoptosis assays

To test whether LPva treatment and/or UPEC infection induced programmed cell death, T24 cells were firstly grown overnight on glass cover slips in 24-well plates.
Cells were then treated with either normal growth medium (control cells) or with LPva aqueous extract at concentrations of 100 µg/ml or 1000 µg/ml for 6, 24 or 72 h. Cells grown for 72 h received fresh medium daily. Following treatment, cells were either stained for apoptosis marker directly (non-infected controls), or were firstly infected with UPEC strain CFT073 for 60 min and then stained. Two different kits were used to investigate apoptosis. These included the Annexin-V-FLUOS staining kit (Annexin assay) and the in situ cell death detection kit (TUNEL assay) (Roche Applied Science, Penzberg, Germany) which were used according to the manufacturer instructions.

The Annexin assay labels phosphatidylserine which is exposed on the cell surface in the early stages of apoptosis (Roche Applied Science). The Annexin-V-FLUOS labelling solution also includes propidium iodide which enables distinction of necrotic (or very late apoptotic) cells due to their impaired membrane integrity (Roche Applied Science). Briefly, medium was removed from non-infected and infected cells and Annexin-V-FLUOS labelling solution was added. Cells were incubated for 15 min at room temperature and then stained with 4',6-diamidine-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich) for 30 s to enable detection of the cell nucleus. Cover slips were then mounted onto microscope slides using ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA, USA).

The TUNEL assay labels DNA strand breaks generated during apoptosis (Roche Applied Science). Briefly, cell culture medium was removed from non-infected and infected cells followed by washing once with PBS. Cells were then fixed with 4% paraformaldehyde (pH 7.4) for 1 h at room temperature and washed with PBS. Permeabilisation of cells was performed using 0.1% Triton X-100 in 0.1% sodium citrate while cells were maintained on ice for 2 min. The permeabilisation solution
was removed and cells were washed with PBS. TUNEL reaction mixture (label solution containing terminal transferase) was then added to each cover slip. A positive control in which DNA strand breaks were induced by the addition of DNase 1 recombinant for 10 min prior to addition of TUNEL reaction mixture was included. One negative control was maintained alongside which was stained with label solution only. All cells, including positive and negative controls, were incubated for 60 min in the dark in a humidified incubator at 37°C and then washed three times with PBS. Cover slips were mounted onto microscope slides as per the Annexin Assay.

In both the Annexin and TUNEL assays, medium was collected and pooled throughout the staining procedures and was thereafter stained according to the appropriate assay protocol to determine whether apoptotic cells were detaching and being removed during this process. Cells were examined with a Leitz-Leica DMRB microscope (Leica, Heidelberg, Germany) using 100× objective and appropriate filter settings.

5.3.4 Total RNA isolation and PCR analysis

The effect of LPva extract and UPEC infection on the expression of a number of genes that mediate attachment and invasion was then investigated. The effects on LPva extract on antimicrobial responses in T24 cells were also investigated by measuring the mRNA expression of antimicrobial peptides. Non-treated control and 72 h LPva-treated cells (100 μg/ml) grown in 24-well plates. Furthermore, levels of β1 integrin mRNA expression were also tested in cells treated with 10 μg/ml LPva for 5 days. To avoid overconfluence during this extended period, treated and non-treated cells were firstly grown in flasks for 2 days, then sub-cultured and seeded
into 6-well plates for a further 3 days of treatment. After 72 h or 5 days of treatment, cells were infected with UPEC strain CFT073 for 60 min. Non-infected cells were maintained alongside as controls. Following infection, bacteria and/or cell culture medium was removed and total RNA was extracted from cells using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Briefly, immediately following removal of cell culture medium, the cell monolayer was disrupted using BLT buffer. The lysate was transferred to a QIAshredder spin column and centrifuged (14 000 rpm, 2 min). Ethanol (70%) was added to the lysate and mixed thoroughly. The sample was then transferred to the RNeasy spin column and centrifuged (10 000 rpm, 15 s). The flow-through was discarded and buffer RW1 was added to the column before centrifuging as before. Flow-through was again discarded and the columns washed twice with buffer RPE by centrifuging (10 000 rpm, 2 min). The spin column was placed in a sterile tube and 50 μl of RNase-free water was added to the column. Thereafter, RNA was eluted by centrifuging the column at 10 000 rpm for 1 min and was then quantified by spectrophotometer using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.). Synthesis of cDNA was performed using the DyNAmo cDNA Synthesis Kit (Finnzymes, Espoo, Finland) as recommended by the manufacturer. Briefly, a reaction mixture was prepared containing 2x RT buffer, primers, reverse transcriptase, RNase-free water and 500 ng of RNA template. To enable reverse transcription, samples were subjected to an incubation program consisting of primer extension (25°C, 10 min), cDNA synthesis (37°C, 30 min) and reaction termination (85°C, 5 min). Real-time PCR for caveolin-1 and β1 integrin, and the antimicrobial peptides human beta defensin 2 (HBD-2) and LL-37, were performed using gene-specific TaqMan
Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) in a Rotor-Gene PCR cycler (Corbett Life Science). 18S rRNA (Applied Biosystems) served as an internal control. Relative expression of the target genes was determined using the \( \Delta\Delta C_t \) method (134). Experiments were performed at least three times independently with triplicate samples for each condition.

5.3.5 Protein extraction and Western blot analysis

Cells seeded into 6-well plates were grown overnight and were given either fresh medium (non-treated control cells) or treated with LPva extract (100 µg/ml) for 24 or 72 h. The levels of \( \beta1 \) integrin protein expression were also tested in cells treated with 10 µg/ml of LPva extract for 5 days. Cells were treated in flasks for 2 days before being sub-cultured and seeded into 6-well plates for a further 3 days of treatment. Following treatment, cells were infected with UPEC strain CFT073 for 60 min. Non-infected cells served as controls. After 60 min, the medium was replaced with fresh medium containing 50 µg/ml gentamicin (Sigma-Aldrich) and cells were incubated for a further 5 h. Plates were then placed on ice, cells were washed three times with ice-cold PBS and lysed with lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 1% IGEPAL CA-630 and 10% glycerol) containing complete protease inhibitor cocktail (1:100, Sigma-Aldrich). The lysates were sonicated for a total of 30 s in 10 s intervals while maintained on ice. The protein concentration was measured using the BCA Protein Assay kit (Pierce, Thermo Scientific, Waltham, MA, USA). Briefly, lysates were diluted (1:10) in PBS. A 50 µl volume of lysate was mixed with 1 ml of BCA reagent solution consisting of BCA protein assay reagent solutions A and B (1:50). Samples were incubated in a water bath at 60°C for 30 min. The absorbance was then read at 562 nm and the protein concentration
was calculated based on a previously established standard curve of protein concentration. Samples were adjusted to equal concentration and boiled in Laemmli sample buffer (Bio-Rad Laboratories) for the detection of β1 integrin. To detect caveolin-1, Laemmli sample buffer was supplemented with 5% β-mercaptoethanol. Samples were boiled at 95°C for 5 min and were stored at –20°C. For Western blot analysis, samples were subjected to SDS-polyacrylamide gel electrophoresis on a 10% Tris-HCl ready gel (Bio-Rad Laboratories). Proteins were transferred to a 0.45 μm Invitrolon PVDF membrane (Invitrogen). The membrane was blocked with 5% milk in Tris-buffered saline with 0.05% Tween 20 for 1 h at room temperature. Primary antibody was added and the membrane was incubated overnight at 4°C, followed by incubation with secondary antibodies for 1 h at room temperature. Antibodies used included goat anti-caveolin-1 (1:500; Abd Serotec, Oxford, UK), mouse anti-β1 integrin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and HRP-conjugated anti-goat antibody (1:1000, R&D Systems, Abington, UK) or anti-mouse antibody (1:30 000, Bio-Rad Laboratories). Proteins were visualized using Super Signal West Pico kit (Pierce). For loading control, membranes were stripped and re-probed with rabbit anti-GAPDH antibodies (1:1000; Cell Signaling Technology, Danvers, MA, USA). Band intensity was quantified using ImageJ (NIH) software and normalised to GAPDH in the same sample. Experiments were repeated at least three times independently.

5.3.6 Bacterial interactions with LPva extract

LPva extract was tested for potential antimicrobial activity against different uropathogenic species in an agar diffusion sensitivity assay. Species tested included

*E. coli* (ATCC 25922), *Proteus mirabilis* (ATCC 29906), *Pseudomonas aeruginosa*
Staphylococcus aureus (ATCC 29213), S. saprophyticus (ATCC 15305) and Candida albicans (ATCC 90028). In short, mid-logarithmic phase cultures were embedded at a final concentration of 0.5 x 10^5 CFU/ml into a thin layer of LB broth with 1% agar and low electrolytes. Holes of 3 mm diameter were made in the agar and a 3 µl aliquot of 1000 µg/ml LPva was added. Plates were incubated overnight at 37°C and the antimicrobial potential of LPva extract was assessed by the diameter of the zone of inhibition around the hole.

To investigate the interaction of LPva with E. coli type 1 fimbriae, UPEC strain CFT073 was grown in LB broth without shaking to promote expression of type 1 fimbriae. The culture was then centrifuged and resuspended in PBS. Bacteria (approximately 10^{10} CFU/ml) were mixed in equal parts with a suspension of Baker’s yeast (Saccharomyces cerevisae, 3% w/v in PBS) and inspected for agglutination. Specificity of the reaction was tested by the inhibitory effect of 5% mannose. The potential interaction of LPva extract with FimH was tested by replacing mannose with LPva extract.

5.3.7 Adherence and invasion assays

The effect of LPva aqueous extract on UPEC adherence and invasion of the uroepithelium were then investigated in comparison to non-treated cells. In both assays, cells were grown in 24-well plates for 72 h without or with 100 µg/ml LPva extract. Cells were infected with UPEC strain CFT073 for 30 min. Bacteria and cell culture medium were then removed and wells were washed three times with PBS to remove non-adherent bacteria. In the adherence assay, cells were then lysed with 1% Triton X-100 in PBS. The lysate was serially diluted and plated on blood agar plates.
Following overnight incubation at 37°C, bacterial counts were made to determine the total number of cell-associated bacteria. Data for LPva-treated cells were normalised to results obtained for non-treated control cells.

To test for invasion, cells were washed with PBS following the 30 min infection period and fresh medium was added. Cells were incubated for a further 90 min to allow bacterial invasion of T24 cells. Cells were washed once with PBS and incubated with fresh medium containing 100 μg/ml gentamicin (Sigma-Aldrich) for a further 60 min. Cells were again washed with PBS and then lysed, serially diluted and plated on agar plates as per the adherence assay. Where medium changes occurred, treated cells received medium supplemented with LPva extract throughout the experiment.

5.3.8 Statistical analysis

Statistical analysis was performed using Wilcoxon-Mann-Whitney tests for comparisons between treatment and infection conditions. Differences were considered significant if $P < 0.05$.

5.4 Results

5.4.1 Apoptosis

LPva extract contains an antioxidant compound, anthocyanin (170) which has been shown to induce apoptosis in both rat epithelial cells (249) and hepatoma cells (215). In view of the role of apoptosis in the innate immune defence against UTI (161), the effects of LPva extract in inducing programmed cell death were therefore investigated. In the Annexin assay, no apoptosis was observed in non-treated control cells (Fig. 5.1A). However following infection, cells stained with both Annexin-V
and propidium iodide, suggesting late stages of apoptosis and necrosis (Fig. 5.1B). Cells treated with LPva (100 µg/ml) stained positively with Annexin-V but not propidium iodide, without and with UPEC infection (Fig. 5.1C and 5.1D, respectively). Annexin-V staining was also seen among cells treated with 1000 µg/ml LPva extract. Cells collected in the medium also stained with apoptosis marker confirming the loss of detached apoptotic cells. Similar results were obtained after both 6 and 24 h of treatment with LPva aqueous extract. In view of the above finding, it is likely that in the viability assays, non-viable LPva-treated cells may have been washed off during the washing procedures, thus leading to fewer non-viable cells left at the completion of the staining process.
Figure 5.1. Apoptosis induced by LPva extract in T24 bladder epithelial cells. The Annexin assay revealed apoptosis in 100 µg/ml and 1000 µg/ml LPva-treated cells. (A) No apoptosis was seen in non-treated control cells however; (B) late apoptotic (stained green with Annexin-V), necrotic cells (stained red with propidium iodide) were detected following UPEC infection. (C) Apoptosis was seen among non-infected 24 h 100 µg/ml LPva-treated cells and (D) following UPEC CFT073 infection. All cells were stained with DAPI (blue) to detect the cell nucleus. Images shown are those following 24 h of treatment however; similar results were seen after just 6 h of treatment.

To verify the induction of apoptosis among LPva-treated cells, the TUNEL assay was employed. As per the Annexin assay, no apoptosis was detected among non-treated control cells. In contrast to the Annexin assay however, TUNEL staining did
not reveal apoptosis among cells treated for 6 and 24 h with 100 µg/ml of LPva extract. Furthermore, in the infection condition, apoptosis could not be confirmed due to the loss of cells during multiple washing steps of the staining procedure. However, the TUNEL assay, which mirrors late stages of apoptosis, revealed apoptosis in all cells treated with 1000 µg/ml of LPva extract for 6 and 24 h (Fig. 5.2A and 5.2B, respectively).

![A B](image)

**Figure 5.2.** Apoptosis induced by LPva aqueous extract in T24 bladder epithelial cells. In the TUNEL assay, apoptosis (stained green with TUNEL reaction) was observed in cells treated with LPva extract (1000 µg/ml) for 6 h (A) and 24 h (B).

Finally, apoptosis could not be detected among cells treated for 72 h with 100 µg/ml of LPva extract using the two assays employed. This went hand in hand with cell viability results from both trypan blue and XTT assays which showed no differences between cells treated with LPva extract compared to non-treated control cells at this time point (data not shown).
5.4.2 Expression of caveolin-1

The expression of apoptosis-promoting caveolin-1 was investigated in non-treated control and LPva-treated (100 µg/ml) cells. Caveolin-1 mRNA expression was found to be upregulated following treatment with 100 µg/ml of LPva aqueous extract compared to non-treated control cells without and with UPEC strain CFT073 infection (Fig. 5.3). Since similar trends were seen following 6, 24 and 72 h of treatment LPva extract, only results following 6 h of treatment are shown in Figure 5.3.

![Graph showing mRNA expression of caveolin-1](image)

**Figure 5.3.** The mRNA expression of caveolin-1 in T24 cells following treatment with LPva extract. Expression of caveolin-1 mRNA was significantly upregulated in cells treated for 6 h with 100 µg/ml LPva extract with (**) \( P = 0.01 \) and without (***) \( P = 0.001 \) UPEC strain CFT073 infection in comparison to non-treated control cells. Similar trends were seen at 24 and 72 h. Values shown are mean ± standard error of the mean from four independent experiments, each containing 3 replicates per treatment condition \( (n = 12) \). Results were normalised to non-treated, non-infected control cells.

Expression on the protein level was then investigated. It was found that increased expression of caveolin-1 occurred in 72 h LPva-treated cells compared to control
cells, with and without infection (Fig. 5.4A and B). This effect was not seen at earlier time points.

**Figure 5.4.** The level of caveolin-1 protein expression in T24 cells following 72 h of treatment with LPva extract. (A) At the protein level, caveolin-1 was significantly upregulated among cells treated for 72 h with LPva extract (100 µg/ml) compared to non-treated control cells, with and without UPEC strain CFT073 infection (*P < 0.05). Data shown are mean ± standard error of the mean from three independent experiments with one replicate per treatment condition (n = 3). Results were normalised to non-treated, non-infected control cells. (B) Representative Western blots for the caveolin-1 protein expressed in non-treated control and LPva-treated cells.
5.4.3 Expression of β1 integrin

Hand in hand with apoptosis, it was observed that LPva-treated cells detached from cell culture dishes more easily than non-treated cells. In view of the roles that integrin proteins play in cell-cell and cell-matrix adhesion (80, 99) and the role of β1 integrin in mediating invasion (66), the effect of treatment with LPva extract on the expression of β1 integrin among T24 cells was therefore investigated. Cells treated with LPva extract (100 µg/ml) for 72 h had significantly decreased mRNA expression of β1 integrin compared to non-treated control cells ($P < 0.01$) (Fig. 5.5). In the presence of infection however, the level of expression was not different between LPva-treated and non-treated control cells (Fig. 5.5).

![Bar graph](image)

**Figure 5.5.** The mRNA expression of β1 integrin in T24 cells following 72 h of treatment with 100 µg/ml LPva extract. The level of β1 integrin mRNA expression was significantly downregulated in LPva-treated cells compared to non-treated control cells (**$P = 0.01$**). However, during infection, the expression between non-treated and LPva-treated cells was not different. Data shown are mean ± SEM, calculated from a total of four independent assays with six replicates ($n = 24$) per treatment condition. Results were normalised to non-treated, non-infected control cells.
To confirm the results seen at the mRNA level, the level of protein expression was investigated. However, the reduction in expression of β1 integrin among non-infected LPva treated cells was not observed at the protein level after 72 h of treatment (Fig. 5.6).

**Figure 5.6.** The protein expression of β1 integrin in T24 cells following 72 h of treatment with 100 µg/ml LPva extract. The level of β1 integrin was not different among LPva-treated cells in comparison to non-treated control cells, without or with UPEC CFT073 infection. Data shown are mean ± SEM, calculated from a total of three independent assays with one replicate (n = 3) per treatment condition. Results were normalised to non-treated, non-infected control cells.

Cells were therefore treated for a longer period to determine if expression of β1 integrin occurred at a later time point. Interestingly however, cells treated with 100 µg/ml LPva extract did not re-attach to cell culture dishes after trypsinisation. Therefore, a 10-fold lower LPva concentration was used over 5 days. After this
treatment period, a significant reduction in β1 integrin mRNA expression was again detected among non-infected LPva-treated cells (Fig. 5.7).

**Figure 5.7.** The mRNA expression of β1 integrin following treatment of T24 cells with 10 µg/ml LPva extract. Reduced expression of β1 integrin was observed after 5 days of treatment with 10 µg/ml LPva-treated cells compared to non-treated control cells (*P < 0.05) but was not observed in the presence of UPEC strain CFT073. Data shown are mean ± SEM, calculated from three independent experiments with three replicates (n = 9) per treatment condition. Results were normalised to non-treated, non-infected control cells.

Since similar levels of mRNA expression were seen among non-treated control and LPva-treated cells in the presence of UPEC strain CFT073, the effects on the expression of protein were consequently investigated in the non-infected condition only. A significant reduction in β1 integrin protein expression was then detected among LPva-treated cells compared to non-treated control cells (Fig. 5.8A & B).
Figure 5.8. The expression of β1 integrin protein in T24 cells following 5 days of treatment with 10 µg/ml LPva extract. (A) Reduced expression of β1 integrin was detected among LPva-treated cells in comparison to non-treated control cells (*P < 0.05). Data shown are mean ± SEM, calculated from three independent experiments with one replicate (n = 3) per treatment condition. Results were normalised to non-treated, non-infected control cells. (B) Representative Western blots for the β1 integrin protein expressed in non-infected, non-treated control and LPva-treated cells.

5.4.4 Antimicrobial activity

In the sensitivity assay, LPva did not produce a zone of inhibition against *E. coli*, *P. mirabilis*, *P. aeruginosa*, *S. saprophyticus* and *Candida albicans*, suggesting that LPva did not have any direct inhibitory activity against these uropathogens. However, a distinct zone of inhibition was observed against *S. aureus*.

The mRNA expression of antimicrobial peptides HBD-2 and LL-37 was similar to that of control cells suggesting that LPva extract did not influence bladder epithelial cell antimicrobial responses (Fig. 5.9)
Figure 5.9. The expression of antimicrobial peptides HBD-2 and LL-37 in LPva-treated T24 cells. mRNA expression of HBD-2 and LL-37 was not affected by 100 µg/ml LPva extract in comparison to non-treated control cells, without and with UPEC CFT073 infection. Expression of mRNA was calculated from a total of 3 independent assays with three replicates (n = 9) per treatment condition. Results were normalised to non-treated, non-infected control cells.

5.4.5 Bacterial adherence and invasion

Type 1 fimbriae are the main adhesins mediating UPEC binding to bladder epithelial cells via host cell mannose residues and FimH at the tip of the bacterial pili (1). LPva extract did not have an effect on the binding of FimH of UPEC strain CFT073 to its receptor, as analysed by agglutination of yeast cells. In line with this finding, LPva extract did not affect UPEC strain CFT073 adherence to T24 cells (Fig. 5.10).

However, a reduction in the number of intracellular bacteria was observed in LPva-treated cells (Fig. 5.10).
Figure 5.10. Level of adherence to and invasion by UPEC strain CFT073 of T24 cells treated with 100 µg/ml of LPva extract. LPva treatment did not affect adherence. However, treatment was associated with a significant decrease in the level of intracellular bacteria (**p = 0.01). Data shown are mean ± standard error of the mean, calculated from three independent assays with six replicates (n = 18) per treatment condition. Results were normalised to non-treated control cells.

5.5 Discussion

In the present study, it was found that treatment with aqueous extract of LPva induced apoptosis in bladder epithelial cells, accompanied by elevated levels of caveolin-1 and reduced expression of β1 integrin. LPva extract did not affect bacterial adherence via type 1 fimbriae and bladder epithelial antimicrobial responses, however, LPva treatment reduced the level of intracellular bacteria compared to non-treated cells.

In vivo, the innate immune response induces apoptosis and exfoliation of superficial bladder epithelial cells when bacterial surface components are detected (161). This is the first study to demonstrate that LPva extract induces apoptosis in human bladder
epithelial cells. These effects may be related to the presence of anthocyanins, antioxidant compounds which have been shown to be apoptosis-inducing (215, 249), in LPva extract (170). In the Annexin assay, findings in the current study revealed apoptosis in T24 cells after 6 and 24 h of treatment with 100 µg/ml and 1000 µg/ml of LPva extract. On the other hand, in the TUNEL assay, apoptosis was observed among cells treated with 1000 µg/ml of LPva extract only. It is likely that the differences between these results may be explained by the different stages of apoptosis that the two assays indicate. Following infection, the Annexin assay also revealed apoptosis among cells treated with 100 µg/ml of LPva extract and among detached cells collected in the medium. While no apoptosis was observed among non-treated cells, the presence of propidium iodide-positive cells following infection indicated necrosis. The lack of propidium-iodide staining among infected cells treated with LPva extract suggested that LPva protected cells under the condition of infection. Since UPEC is known to induce apoptosis (161), propidium iodide-positive cells most likely represent cells at the terminal stage of apoptosis which remained in the absence of in vivo phagocytic activity (184). The lack of apoptotic cells observed among cells treated with LPva for 72 h may be explained by the detachment and loss of dead cells at earlier time points, leaving only viable cells. Collectively, the present findings on the induction of apoptosis among cells treated with LPva extract indicate the presence of active compounds in the extract. However, further research is required before it can be determined which compound or compounds are responsible for the effects seen here and the concentration at which these effects are exerted.
Caveolin-1 has many functions at the cell surface including involvement in lipid transport, membrane traffic and cell signalling (133). Interestingly, elevated levels of caveolin-1 have been proposed as a marker of apoptosis in macrophages (77) and have furthermore been shown to ‘sensitise’ T24 bladder epithelial cells to other apoptosis-inducing stimuli (132). Type 1 fimbriae may function as stimuli that induce this host response (161). In the present model, it is therefore likely that UPEC infection and consequent adherence of type 1 fimbriae further promoted apoptosis among cells treated with LPva extract.

Results from this study revealed that LPva extract does not elicit antimicrobial activity among T24 cells and does not exhibit effects against most of the uropathogens tested. However, its antimicrobial activity against *S. aureus* further indicated the role of anthocyanins as these compounds have previously been shown to have inhibitory activity against this species but not others such as *E. coli* and *P. aeruginosa* (129).

LPva extract did not affect bacterial adherence to bladder epithelial cells which was not unexpected considering that the extract did not interfere with the binding of type 1 fimbriae to its receptors. Despite no effect on adherence, however, a significant reduction in the level of intracellular bacteria was observed in LPva-treated cells in comparison to non-treated control cells. Invasion of superficial bladder epithelial cells (161) and formation of intracellular bacterial communities (19) is a key virulence property of UPEC and has been associated with recurrent infection (162). Both bacterial virulence factors and host receptors contribute to UPEC invasion (162). Additionally, type 1 fimbriae may enable UPEC invasion of exposed deeper
layers of the epithelium (161) which may be facilitated by their binding to β1 integrin receptors (66). Based on the findings in this study, it is possible that following LPva-induced apoptosis and consequent exfoliation of dead cells, the decreased expression of β1 integrin could protect deeper layers of the uroepithelium from UPEC invasion. It could be speculated that down-regulated expression of β1 integrin accounted for the observation of reduced invasion. However, under the experimental conditions used for infection assays, reduced expression seen at the mRNA level could not be confirmed on the protein level at the same time point. In vivo, a down-regulation in the expression of this protein may, however, play a role in reduced invasion of bladder epithelial cells.

In conclusion, this study for the first time, demonstrated the effects of aqueous extract of LPva in a cell culture model of the human uroepithelium and UPEC infection. The findings revealed that the aqueous extract from this medicinal plant induced apoptosis and reduced invasion of bladder epithelial cells, mechanisms which may both help to eliminate infection in the urinary tract. The results of this study collectively provide indications that LPva extract has potential applications against UTI which may be of increased significance in the face of increasing incidence of infection caused by antibiotic resistant UPEC.
CHAPTER 6

6.1 General Discussion and Closing Remarks

The intestinal microbial community contributes significantly to the health of the host (175) but may also be a reservoir for pathogenic bacteria causing intestinal or extraintestinal diseases (198). Indeed, gut-origin E. coli is one of the most common causative agents of extraintestinal infections such as septicaemia and UTI (198). Such infections may be suffered by millions of people per year and may be life-threatening to the patient (198). Understanding the mechanisms of action and virulence factors that are employed by these bacteria is therefore of high importance.

Such knowledge may enable development of more targeted treatment options leading to better prevention, management and/or treatment of infection.

For gut E. coli to translocate and cause septicaemia, or colonise the urinary tract to cause infection, certain virulence factors are required to help the bacterium to adhere to and invade the host epithelium and/or survive the host immune defence. Whilst studies on virulence properties associated with strains causing septicaemia have shown most belong to phylogenetic group B2 and carry a high number of virulence genes, some E. coli strains belonging to non-B2 phylogenetic groups may still cause septicaemia and yet not carry many of these virulence genes. An example of this was found in the present study where E. coli strains that were highly efficient in adhesion and translocation belonged to groups B1 and D and carried very few virulence genes. Furthermore, some of these genes were not consistently found in all strains tested. Of those found among the TEC strains in this study, type 1 fimbriae might be a significant contributing factor in the translocation process by mediating adhesion to and invasion of the gut epithelium. However, since this study only investigated
virulence factors at the gene level, their expression and role in translocation cannot be confirmed without further investigation. Furthermore, in view of the finding that many known virulence genes that are associated with E. coli pathogenicity were absent among the TEC strains, the involvement of other, and perhaps novel virulence factors must be considered when studying the translocation process. In this respect, a shortfall of this thesis could be a lack of investigation into identifying the presence of such virulence factors. Although beyond the scope of the present study, future directions investigating the pathogenesis of TEC strains may involve the use of molecular techniques such as signature tagged mutagenesis or transposon mutagenesis to identify genes involved in translocation. For instance, random insertion of a transposon into the genome of TEC strains, leading to gene interruption, changes in its expression and loss of translocation ability, may enable identification of virulence factors that are involved in this process. However, such an approach requires a specific, highly reproducible and yet easy to perform test assay, as the adhesion and translocation assays may in some cases yield variability and are dependent on the type of cell line used.

In the face of infection, the host initiates an immune response (12, 59, 136). The success and further progress of the infection depends on the ability of the invading microbe to overcome such defences. In the case of septicaemia and UTI, E. coli strains can survive despite a multitude of host defence mechanisms, aided by virulence properties that enable the bacterium to either mimic the host molecules and evade the immune defence system or, protect itself from phagocytosis. An example of this was seen in this study where human and pig-origin TEC strains capable of synthesising capsule induced an elevated immune response but still translocated with high efficiency. This was in contrast to a rat TEC strain not carrying capsule
synthesis genes which induced elevated immune responses and although able to adhere as efficiently, did not translocate to the same degree. These findings suggest that the translocation of bacteria is more dependent on their ability to survive the immune system rather than their ability to cross the human gut epithelium. Alternatively, a combination of both of these factors may have contributed to higher efficiency of translocation among certain strains. An interesting factor was that the rat TEC strains not carrying capsule synthesis genes were shown in an in vivo study to efficiently translocate in rats and in pigs (119) which may indicate host-specific immune responses played a role in the differences observed in comparison to the present study. Furthermore, it is noteworthy that most E. coli strains shown to translocate and/or isolated from MLNs or blood of the host, including TEC strains used in this study, were found to translocate in models where the host immune system was compromised (e.g. haemorrhage or 48 h starvation) (14, 116, 118, 135, 168). Although both pathogenic and non-pathogenic bacteria might have equal opportunity to translocate in these conditions, it is likely that only those capable of resisting the immune system survived and were isolated. Whether the bacterial capsule has any role in translocation, or only in the survival of TEC strains remains to be elucidated. However, in view of the high prevalence and possible roles of the bacterial capsule in the pathogenesis of septicaemic E. coli, it is possible that it may be a potential treatment target among patients with gut-origin sepsis. Drugs targeting disruption of the bacterial capsule and/or its synthesis may provide the host immune system with increased opportunity to effectively defend against such bacteria.

The high recurrence rates and severe outcomes that may result from UTI among certain patients have attracted researchers to explore the virulence properties
associated with \textit{E. coli} persistence in the host urinary tract. In this thesis, the diverse geographic areas from which isolates were obtained was a strength of the study and the conclusions drawn apply to wider communities. The significant differences between countries, not just in the prevalence of virulence factors, but also in the level of antibiotic resistance highlight the complexities in the treatment of UTI and urosepsis. Many studies have shown high prevalence and functional roles of adhesins such as type 1 fimbriae and P pili in UPEC colonisation. Although there were variations in the prevalence of \textit{fluA}_{CFT073} or \textit{fluB}_{CFT073} in different countries in this study, the high prevalence of the \textit{flu} gene among UPEC isolates from children suggests roles in the pathogenesis of these bacteria. Since gastrointestinal colonisation of \textit{E. coli} occurs during the early stages of life, it is possible that gut flora of children, at least in countries studied here, serves as a long term source of \textit{E. coli} carrying Ag43 genes. If this hypothesis is true, it warrants a longitudinal study exploring the prevalence and persistence of \textit{E. coli} strains carrying Ag43 in the gut flora of children and probably adults. It may be considered that a shortfall of this study was investigation of only two Ag43 allelic variants among UPEC strains. This could be justified by the fact that of the 5 known variants of the \textit{flu} gene, only \textit{fluA}_{CFT073} and \textit{fluB}_{CFT073} have been shown to have functional roles in \textit{E. coli} causing UTI (230). The roles of other variants in UPEC pathogenesis remain to be fully investigated. Nonetheless, the overall high prevalence of Ag43 among these isolates and an association with resistance to beta-lactam antibiotics suggests a role for at least some variants in UPEC pathogenesis and in the development of resistance. Since this association was observed among isolates from a number of geographically distant countries, it is possible that this combination in virulence factor and resistance may be common among UPEC. Another possibility is that the
development of resistance due to antibiotic selective pressure may have been promoted among isolates equipped to persist. Despite having protective and colonisation-promoting roles, results from this study indicated that curli and cellulose do not play dominant roles in the pathogenesis of UPEC in either of the patient groups investigated. Moreover, since lack of expression of these virulence factors was associated with resistance among certain isolates, a trend which has not previously been described, it is possible that expression of these virulence factors had been lost at the expense of development of resistance. Collectively, these results raise the question on which UPEC virulence factors play a more important role in promoting the development of resistance and which virulence factors can be lost at the expense of developing resistance. From this point of view, it is possible that factors promoting the development of resistance are those which promote colonisation and/or long-term survival and may be the most suitable treatment targets to combat both infection and rising levels of resistance.

Antibiotic resistance among bacteria not only indicates greater challenge in treating infected patients but also provides UPEC with increased opportunity to persist in the bladder and ascend to the kidneys to cause pyelonephritis and other associated diseases such as renal scarring and septicaemia. The high incidence of resistance to multiple classes of antibiotics among \textit{E. coli} isolates causing UTI in children and pregnant women, especially in countries where antibiotics are used inappropriately, is of great concern. Ability to buy antibiotics from private sellers, with lack of medical knowledge or advice as to the most appropriate antibiotic to treat the infection, the correct concentration of the active substance and the correct length of treatment places patients in significant danger of developing resistance and further complications, as described above. These concerns may also extend to the
availability of antibiotics over the internet. The severe and potentially life-threatening consequences of upper UTI cannot be taken lightly among these high-risk patient groups. Therefore, stricter, more regulated and carefully implemented measures in the use of antibiotics in these countries are the first step toward controlling the spread of resistance genes among bacteria. This recommendation also includes the need for more responsible use of antibiotics, the need for new antibiotics and/or alternative treatment options. In this respect, a study was undertaken in this thesis which endeavoured to investigate the role of traditional medicinal plants in the treatment of UTI caused by *E. coli*.

The use of plant products to treat human conditions is not a new concept. Around the world, many examples can be found where traditional medicinal plant products are used and claimed to be successful in treating many different ailments. Investigation into the mechanisms of action of these medicinal plants can be undertaken in a laboratory setting to verify the purported effects and if successful, may pave the way for the development of commercially available products. In this thesis it was shown that the aqueous extract of the Malaysian medicinal herb, LPva, had a number of protective effects which included inducing apoptosis and reducing bacterial invasion of bladder epithelial cells. These effects however, were only tested in one bladder epithelial cell line. Thus, the current data does not allow for firm conclusions to be made on whether LPva may indeed prevent and/or have an impact on UTI in the human body. Furthermore, while the effects seen in this thesis indicate that certain antioxidant compounds may have been responsible, it cannot be ruled out that other compounds may also have the same effect and/or that the effects seen were mediated by more than one active compound present in the extract. Despite these shortfalls,
the significant findings contributed by this study open a window for further investigation. Future directions may include testing the effects of LPva in other cell lines, including primary cells, and in laboratory animal models such as a rat model of UTI. It may also be possible to test whether LPva has anti-UTI effects among volunteer women at risk of recurrent infection and/or those suffering UTI. Additionally, identification of the active compound(s) responsible for the effects seen in this study may contribute to development of products aimed at preventing and/or treating UTI.

In conclusion, the importance of colonisation, adhesion and persistence in the pathogenesis of septicaemic and uropathogenic *E. coli* are highlighted in this thesis and indicate that virulence factors associated with these mechanisms may be good targets in the development of treatment strategies against such bacteria. Targeting certain virulence factors may provide the host defence system with the opportunity to prevent and/or slow down the rate of translocation or progression of UTI. High prevalence of antibiotic resistance among isolates causing UTI in young children and pregnant women also suggests the need for alternative treatment strategies. Findings on the effects of LPva extract collectively show that medicinal herbs may be appropriate avenues of investigation in the fight against extraintestinal infections caused by *E. coli*.  

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REFERENCES

The referencing format of this thesis is in accordance with that of the Journal of Clinical Microbiology, published by the American Society for Microbiology.


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131. **Li, Y. F., S. Poole, F. Rasulova, A. L. McVeigh, S. J. Savarino, and D. Xia.** 2007. A receptor-binding site as revealed by the crystal structure of CfaE, the colonization factor antigen I fimbrial adhesin of enterotoxigenic *Escherichia coli*. J. Biol. Chem. 282:23970–23980.


## APPENDIX A

Table: Prevalence of virulence genes and phylgenetic groups among septicaemic and uroseptic *Escherichia coli* isolates. From Ramos, 2007 (187).

<table>
<thead>
<tr>
<th>Virulence gene</th>
<th>Function</th>
<th>Septicaemic (n = 40)</th>
<th>Uroseptic (n = 30)</th>
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<tbody>
<tr>
<td>aah (orfA&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>Autotransporter adhesin</td>
<td>0</td>
<td>10</td>
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<tr>
<td>aidA (orfB)</td>
<td>Diffuse adherence</td>
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<td>0</td>
</tr>
<tr>
<td>AIDA&lt;sup&gt;c&lt;/sup&gt; (orfB&lt;sup&gt;C&lt;/sup&gt;)</td>
<td>Diffuse adherence</td>
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<td>3</td>
</tr>
<tr>
<td>afalatraBC</td>
<td>Dr-binding adhesins</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>bmaE</td>
<td>M-agglutinin subunit</td>
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<td>97</td>
</tr>
<tr>
<td>cdt</td>
<td>Cytolethal distending toxin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cdtB</td>
<td>Cytolethal distending toxin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>chuA</td>
<td>Haeme transport</td>
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<td>97</td>
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<tr>
<td>cnf1</td>
<td>Cytotoxic necrotizing factor</td>
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<tr>
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<td>Colicin V</td>
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<tr>
<td>EAST1</td>
<td>EAggEC heat-stable enterotoxin</td>
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<tr>
<td>eltA(LT)</td>
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<td>faeG(F4)</td>
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<td>fanC (F5)</td>
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<td>0</td>
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<td>fimH</td>
<td>D-mannose specific subunit, type 1 fimbiae</td>
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<td>93</td>
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<td>F1C fimbriae</td>
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<td>Yersiniabactin receptor</td>
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<td>α-haemolysin</td>
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<td>ibeA</td>
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<td>ipaH</td>
<td>Invasion plasmid antigen</td>
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<td>Novel siderophore receptor</td>
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<td>30</td>
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<td>iroN&lt;sub&gt;E.coli&lt;/sub&gt;</td>
<td>Novel catecholate receptor</td>
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<td>iss</td>
<td>Serum surviva</td>
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<td>iutA</td>
<td>Ferric aerobactin receptor</td>
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<td>nfaE</td>
<td>Nonfimbrial adhesin I assembly and transport</td>
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APPENDIX A continued

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<tr>
<th>Virulence gene</th>
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<th>Uroseptic (n = 30)</th>
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<td>Porcine adhesion/ effacement associated</td>
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<td>0</td>
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<td><em>PAI</em></td>
<td>Pathogenicity- associated island</td>
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<td>73</td>
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<td>P fimbriae structural subunit</td>
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<td><em>papC</em></td>
<td>P fimbriae chaperone</td>
<td>47</td>
<td>87*</td>
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<td><em>papG</em> allele II</td>
<td>Pyelonephritis-associated <em>papG</em> variant</td>
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<td>Universal primer for CNF</td>
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<tr>
<td><em>yjaA</em></td>
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**Phylogenetic group**

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<td>A</td>
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<td>B1</td>
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<tr>
<td>B2</td>
<td>65</td>
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<tr>
<td>D</td>
<td>25</td>
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*Prevalence of gene among uroseptic *E. coli* significantly more (*P < 0.0001*) than among septicaemic *E. coli*. 
Figure: Cumulative number of translocating *E. coli* strains using Caco-2 cell monolayers in a two-compartment translocation model over a period of 2 h. The translocation of TEC strains HMLN-1 and PC-1 was more than TEC strains KIC-1 and KIC-2 at 60 and 120 min (*P < 0.001). TEC strain PC-1 translocated significantly more than HMLN-1 at 120 min (**) *P < 0.001). TEER = trans-epithelial electric resistance. All values are mean ± SD. Data from Helen Ison, reported in Katouli *et al.*, 2009 (119).
Figure: Protein sequence alignments of flagellin of TEC strains (HMLN-1, PC-1, KIC-1, KIC-2), non-TEC strain 46-4 and control strain (S. typhimurium (FLIC-SALTY)).

**APPENDIX C**

KIC-2  MAQVINTNLSLLTQNNLKSNQSSLSSSAIERLSSGLRINSKDDAAGQAIAANRFTANIKG 60
FLIC_SALTY MAQVINTNLSLLTQNNLKSNQSSLSSSAIERLSSGLRINSKDDAAGQAIAANRFTANIKG 60
46-4  MAQVINTNLSLLTQNNLKSNQSSLSSSAIERLSSGLRINSKDDAAGQAIAANRFTANIKG 60
KIC-1  MAQVINTNLSLLTQNNLKSNQSSLSSSAIERLSSGLRINSKDDAAGQAIAANRFTANIKG 60
PC-1  MAQVINTNLSLLTQNNLKSNQSSLSSSAIERLSSGLRINSKDDAAGQAIAANRFTANIKG 60
HMLN-1 MAQVINTNLSLLTQNNLKSNQSSLSSSAIERLSSGLRINSKDDAAGQAIAANRFTANIKG 60

KIC-2  LTQASRNANDGISVAQTTEGALNEINNLQRIRELTVQATNGTNSDSDLSSIQAEITQRL 120
FLIC_SALTY LTQASRNANDGISVAQTTEGALNEINNLQRIRELTVQATNGTNSDSDLSSIQAEITQRL 120
46-4  LTQARNANDGISVAQTTEGALNEINNLQRIRELTVQATNGTNSDSDLSSIQDEIKSRL 120
KIC-1  LTQARNANDGISVAQTTEGALNEINNLQRIRELTVQATNGTNSDSDLSSIQDEIKSRL 120
PC-1  LTQARNANDGISVAQTTEGALNEINNLQRIRELTVQATNGTNSDSDLSSIQDEIKSRL 120
HMLN-1 LTQARNANDGISVAQTTEGALNEINNLQRIRELTVQATNGTNSDSDLSSIQDEIKSRL 120

************:****:**.**:*.::***************************:****
APPENDIX C continued

KIC-2   EEIDRVSEQQTQFNGVKVLAENNEMKIQVGANDGETITINLAKIDAKTLGLDGZNIDG-AQ 179
Flic_Salty   NEIDRVSGQTQFNGVKVLAQNITLTIQVGANDGETIDIDLQINSQTLGLDLNQ---Q 177
46-4   DEIDRVSGQTQFNGVNVLAKDGSMKIQVGANDGQITIDLKKIDSDTLGLSGFNVNGGGA 180
KIC-1   DEIDRVSGQTQFNGVNVLAKDGSMKIQVGANDGQITIDLKKIDSDTGLGLSGFVNGGGA 180
PC-1   DEIDRVSGQTQFNGVNVLAKDGSMKIQVGANDGETIDLKKIDSDTNLVAGFNVNGGGE 180
HMLN-1   DEIDRVSGQTQFNGVNVLAKDGSMKIQVGANDGETIDLKKIDSDTNLVAGFNVNGGGE 180

:****** ******:***:**:::. :.********:** *:*::*:.*:*:*:::* :*::

KIC-2   KATGSDLISKFKATGTNDYQINGT-----------------------------DNYTVNVD 211
Flic_Salty   KYKVSDTAATVGYADTIALDNS-----------------------------TFKASATG 209
46-4   VANTAASKDLVAANATVGNKYT-VS---AGYDAAKASDLLAGVS--DGDTVQATINNG 234
KIC-1   VANTAASKDLVAANATVGNKYT-VS---AGYDAAKASDLLAGVS--DGDTVQATINNG 234
PC-1   TANTAATLKMVGLKLDNTGVTTAGVNYIADKAVASSTDILNAGVGSKVSTEADVG 240
HMLN-1   TANTAATLKMVGLKLDNTGVTTAGVNYIADKAVASSTDILNAGVGSKVSTEADVG 240

:****** ******:***:**:::. :.********:** *:*::*:.*:*:*:::* :*::

KIC-2   SGVVQDKDGKQVYVSTADGSLTSSDTQFKIDATKLAVAKDLAQGKNKIVYE---IEFT 268
Flic_Salty   LGGTDAQKDGLKFDTTKGYAKVTGTTGKDGYYEVSVDKNTGEVTLAGG---ATSP 266
46-4   FGTAASAT--NYKYSASKSADTSTSAADVQKYLTPGVDATKGTITIDG----SAQ 288
KIC-1   FGTAASAT--NYKYSASKSADTSTSAADVQKYLTPGVDATKGTITIDG----SAQ 288
PC-1   FGAAAPGTPVEYTHKTNTYAS-ASVDAQLAAFLNPAGGTTAATVSIAPTQEQ 299
HMLN-1   FGAAAPGTPVEYTHKTNTYAS-ASVDAQLAAFLNPAGGTTAATVSIAPTQEQ 299

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<td>NTG----TVAIDAKNGKLTANVDGKAVEFTISGSTDTSATVAPTTALYKN---- 318</td>
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<td>DVQISSDGKITSSN-GDKLYIDTTGRLTKNGFSASLTEGRSLSITLAANNTKATTIDIGGTS 347</td>
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<td>KIC-1</td>
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<td>PC-1</td>
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<td>HMLN-1</td>
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<td>KIC-2</td>
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<td>---DGGLAVKVGDDYYSATQNKDGSIISINTGTKYATADDGSKTALNKLIAGADGKTENVSG 377</td>
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