Detection of intracellular uropathogenic *Escherichia coli* during an *in vitro* infection of bladder uroepithelial cells

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Abstract

Introduction: Urinary tract infection (UTI) is the most common bacterial infection in women. *Uropathogenic Escherichia coli* (UPEC) cause ~80% of all community-acquired UTIs. Although UTIs are traditionally classified as acute infections, the recurrence-frequency is relatively high. Recurrent UTIs are conventionally thought of as reinfections, but recent studies have shown that UPEC-strains use complex pathogenic pathways to accomplish infection of and persistence in the bladder. They do that by establishing intracellular bacterial niches.

Aims: The first aim of this study is to be able to detect living intracellular extended spectrum beta lactamase (ESBL)-producing *Escherichia coli* in an *in vitro* infection of uroepithelial bladder cells. Secondly, this study aims to optimize an *in vitro* model of infection to detect intracellular bacteria.

Methods: Green fluorescent protein (GFP)-expressing pLMB449-plasmid was isolated and introduced into an ESBL-producing UPEC strain (E7). Afterward, several infection models were set up using 5637 bladder cells for an *in vitro* infection and evaluated using fluorescence microscopy and flow cytometric analysis.

Results: E7 was successfully transformed with pLMB449-plasmid and kept its previous pattern of resistance. GFP showed a strong expression and through this, intracellular bacterial communities (IBCs) were observed as well as individual intracellular bacteria. E7 treated with ineffective antibiotic (ceftibuten) became filamentous and gave an indication of having a reduced virulence.

Conclusion: The transformation of pLMB449-plasmid into E7 was successful and the bacteria kept its previous pattern of resistance, gained resistance to gentamicin, and retrained its morphological plasticity in the presence of ineffective antibiotic. The microscopic evaluation of *in vitro* infection models demonstrates that the 6 hours models of infection are convenient to induce intracellular bacteria.
**List of abbreviations**

ASB - Asymptomatic bacteriuria  
CFU - Colony-forming unit  
DMEM - Dulbecco’s modified Eagle’s medium  
ESBL - Extended spectrum β-lactamase  
FBS - Fetal bovine serum  
FS - Forward scatter  
GFP - Green fluorescent protein  
IBC - Intracellular bacterial community  
LB - Luria broth  
MIC - Minimal inhibitory concentration  
MOI - Multiplicity of infection  
PBS - Phosphate buffered saline  
PMN - Polymorphonuclear neutrophil  
QIR - Quiescent intracellular reservoir  
SS - Side scatter  
UPEC - *Uropathogenic Escherichia coli*  
UTI - Urinary tract infection
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1. Introduction

1.1 Urinary tract infections

1.1.1 Disease description
Urinary tract infection (UTI) is an infection in the urinary tract (urethra, bladder, ureters, or kidneys) [1] and includes several types of infections of varying severity, ranging from asymptomatic bacteriuria (ASB) to life-threatening urosepsis [2]. UTIs can be divided into asymptomatic or symptomatic, lower (cystitis) or upper (pyelonephritis), sporadic (initial) or recurrent, uncomplicated or complicated and community acquired or nosocomial [2]. Nevertheless, there are certain features shared by the different states of the spectra, including bacterial colonization of the urine and mucosa, bacterial growth, and possible invasion of the cells and underlying tissues with local and/or general symptoms as a result. The bacteria originate with few exceptions from the patients’ own normal flora [2]. The bacteria enter the urinary tract either by ascending via urethra [1-3], or via the bloodstream [3].

1.1.2 Epidemiology
UTI is the most common bacterial infection in women [2,4], and is associated with considerable morbidity, mortality, and economical costs [1,3,5,6]. In a pregnant woman, UTI raises the risk of maternal pyelonephritis [1], prematurity, and fetal death, while it is, among elderly patients, associated with impaired kidney function and kidney failure [4]. Financially, detection and treatment of community-acquired UTIs cost the United States an estimated annual sum of $1.6 billion [4]. UTI is more common in women than men at all ages [1,4], except among infants [2,4]. It is estimated that one in two women will suffer from at least one symptomatic episode of UTI during their lifetime [2,4]. Almost 10% of women over 18 years old experience at least one episode every year [2]. In 2007 in the USA, UTIs accounted for 10.5 million ambulatory visits, corresponding 0.9% of all ambulatory visits. ASB in women has an overall prevalence of 3.5%, and it increases with age [1,2], and following sexual intercourse [1,7]. Hospital-acquired UTI in catheterized patients is the most common type of nosocomial infections [8], and account for one third of all nosocomial infections [1].

Although UTIs in young, healthy, sexually active women are traditionally classified as acute infections, the recurrence-frequency is relatively high. Approximately 30% (27-44%) of women with acute cystitis experience another episode of UTI (recurrent UTI; rUTI) within 6 months [1,2,9], and 9.2% of women and 5.7% of men suffer from a recurrent pyelonephritis within 12 months after
the first infection [1], despite adequate antibiotic therapy and negative follow-up urine cultures [5,9]. The risk of recurrence increases with the number of recurrences. Moreover, a major risk factor for UTI is a history of a previous episode of UTI [1].

1.1.3 Bacteriology

*Uropathogenic Escherichia coli* (UPEC), a gastrointestinal bacteria, cause ~80% of all uncomplicated, community-acquired, UTIs, whereas *Staphylococcus saprophyticus*, a bacteria commonly present in the female genital tract, causes approximately 15% [2,3]. *E. coli* and *S. saprophyticus* are primary pathogens [2], and the remaining 5-10% are caused by secondary pathogens such as *Klebsiella* species, *Enterococcus faecalis*, *Proteus*, and *Pseudomonas* species especially *Pseudomonas aeruginosa* [2,3,9]. *Klebsiella*, *E. faecalis*, and *Proteus* originate from the gastrointestinal tract, whereas *Pseudomonas aeruginosa* is found in soil, water and skin flora [11]. In patients with complicating factors, such as urinary catheters, hospitalization, diabetes mellitus, and spinal cord injuries, secondary pathogens represent up to 50% of all isolates from urine sampled from patients with UTI [2,9].

1.1.4 Pathogenesis

Urine acts as a passable medium for bacterial growth, which could explain why many bacteria frequently colonize the epithelium in the urinary tract [1]. It is thought that the majority of UTIs arise after bacterial colonization of the vaginal introitus and/or periurethral tissues. These bacteria, often presented in the fecal flora, ascend later to the bladder initiating a host immune response [5]. The superficial uroepithelial cells (umbrella cells) express Toll-like receptor 4 (TLR-4) and CD14. Together they recognize lipopolysaccharide from the outer membrane of the bacteria and initiate an innate host immune response [9]. The host response consists of production of inflammatory cytokines, neutrophilic infiltration into the infected tissue, upregulation of nitric oxide synthase by polymorphonuclear neutrophils (PMNs) leading to high levels of nitric oxide, and exfoliation of the superficial uroepithelial cells [9].

Recurrent infections are conventionally thought of as reinfections through recolonization of the bladder by bacteria that have persisted in the vaginal, periurethral, or fecal flora [9]. However, the majority of rUTIs are caused by bacterial strains that are genetically or phenotypically identical to the strains associated with the initial infection [9], especially rUTIs caused by UPEC-strains [5,12]. Moreover, bladder biopsies from several abacteruric women with recurrent urinary tract symptoms revealed bacterial colonization when cultured [13].
Recent studies have shown that UPEC-strains use complex pathogenic pathways to accomplish infection of and persistence in the bladder despite robust innate host immune response and sufficient antibiotic therapy [14]. They do that by establishing intracellular bacterial communities (IBCs) with a lot of properties shared with extracellular biofilms [5,9]. IBC-formation by UPEC in bladder epithelial cells has been observed in both mice [14,15] and in humans [5].

1.1.4.1 Intracellular bacterial niches
UPEC express an important virulence factor, type 1 pili, by which it may adhere to and invade superficial uroepithelial cells in the bladder. The adhesion is mediated by FimH adhesin, located at the distal tip of type 1 pili [16], that recognize and bind to mannosylated residues on uroplakin Ia-proteins of umbrella cells [5,9]. Type 1 pili can also mediate adhesion to and invasion of the superficial facet cells by binding to α3β1 integrins also located on the superficial bladder cells [17]. After binding to the umbrella cells, UPEC can invade the host cells one to three hours postinoculation [9,15].

Once intracellular, the bacteria start dividing rapidly during the first 6-8 hours postinoculation [9,15]. In this phase the bacteria have a doubling time of approximately 30 minutes [9]. After 6 hours of infection, UPEC form early IBCs, where the bacteria are loosely packed and still rod-shaped. During the next 6 hours, the doubling time of the bacteria increases to 60 minutes and mid-IBCs are formed with tightly packed cockoid bacteria [9,15]. Twelve to sixteen hours post infection, filamentous UPEC detach from late IBCs and escape from the umbrella cells in a process called ‘fluxing‘. The escaping bacteria can then invade other bladder epithelial cells and start another round of IBC-formation [9,15,18]. Filamentous UPEC are more resilient to killing and phagocytosis by PMNs [14,18].

The biofilm-like IBCs mediate prolonged persistence in the bladder through evasion of the immune system and antibiotic resistance [9,18]. The uroplakin coating the plasma membrane of the umbrella cells mediate evasion of the innate immune system by denying the inflammatory cells access into the cell [9]. The resistance to antibiotic agents in the IBCs is due to the impermeable biofilm-like matrix surrounding the intracellular bacteria and the slow growth rate of the bacteria in mature IBCs [9].
In late stages of acute infection, the superficial umbrella cells exfoliate into the lumen, which leads to exposure of the underlying transitional epithelial cells for UPEC invasion [17,18]. This, in turn, leads to acquisition of quiescent intracellular reservoirs (QIRs) [15,17,18]. The bacteria can remain undetected in these QIRs for months, resisting antibiotic treatment, evading the innate immune system, and can be a source of rUTIs [17,18]. Furthermore, bacterial cells in QIRs are metabolic inactive, but can revert to an active state leading to recurrent infection [15].

1.2 Extended Spectrum β-lactamase (ESBL)-producing bacteria

The main mechanism of resistance for Enterobacteriaceae, such as E. coli, against β-lactam drugs is through β-lactamases [19]. β-lactamases are enzymes that hydrolyze β-lactam-antibiotics. β-lactamases can be classified into four classes (A-D) [20] of which, the most common class is class A, SHV-1 (sulphhydryl variable) and TEM-1(Temoneira), penicillinases. These have very little effect on cephalosporins, but simple point mutations may result in the acquisition of the ability to inhibit third generation cephalosporins. These β-lactamases are referred to as extended spectrum β-lactamases (ESBLs) [20]. ESBLs can mediate resistance against all β-lactam antibiotics except carbapenems and cephemycins [19]. The prevalence of ESBL-producing bacteria has increased during the last decades [21]. In addition, most strains producing ESBL are K. pneumoniae and E. coli, which are common uropathogens [21,22].

ESBLs can be troublesome when encoded by plasmids. In that case the resistance is transferable and can be transferred between different Enterobacteriaceae-species [19]. The options of treatment of ESBL-producing bacteria are limited due to co-resistance to other antibiotic classes [22]. An initial ineffective treatment of sepsis caused by ESBL-producing bacteria is associated with increased mortality [23]. Lastly, treatment of ESBL-producing UPEC-strains with ineffective antibiotics may induce filamentation of the bacteria, which in turn alter the innate immune response [24].

2. Aims

The first aim of this study is to be able to detect living intracellular ESBL-producing E. coli in an *in vitro* infection of uroepithelial bladder cells.

Secondly, this study aims to optimize an *in vitro* model of infection to detect intracellular bacteria.
3. Material and Methods

3.1 Human uroepithelial bladder cancer 5637 cells
Epithelial bladder cancer cells (5637; ATCC HTB-9) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen Ltd., Paisley, UK), 2 mM L-glutamine (Invitrogen Ltd., Paisley, UK), and 1 mM non-essential amino acids (Invitrogen Ltd., Paisley, UK) at 37°C in a 5% CO₂ atmosphere and subcultured (passage 4-11) when confluent. Prior to infecting the cells, the cell culture medium was replaced with DMEM supplemented with 2% FBS, instead of the 10% FBS used during cell culturing.

3.2 Bacteria isolates
Two different E. coli strains were used. The ESBL-producing E. coli (E7) was originally isolated from a patient with urosepsis at Örebro University hospital, Sweden. The other E. coli strain, a laboratory strain (DH5α), carrying the pLMB449-plasmid was used to propagate the plasmid. Both isolates were grown on tryptic soy agar (TSA) plate (Becton, Dickinson and Company, Sparks, MD) with or without the presence of gentamicin (10 µg/mL), incubated for 24 hours at 37°C. E7 was grown on TSA plate without gentamicin prior to electroporation.

3.3 Isolation and transformation of the pLMB449-plasmid
The pLMB449-plasmid (kindly provided by Prof. Philip Poole at University of Oxford) carries three important elements (Fig. 1.), Gfpmut3-1, which encodes for GFP-transcription, GmR that confers resistance to gentamicin and also acts as a reporter for Gfpmut3-1, and a common promoter, ptac, which is a fusion of the tryptophan and Lac promoters. Other elements on the pLMB449-plasmid are Rep-genes, which enhance replication of the plasmid, and Mob-genes, which are elements involved in the mobilization of the plasmid. Furthermore, the plasmid carries the mCherry-gene, which encodes for transcription of a red fluorescent protein.
3.3.1 Isolation of the plasmid

Purification of the plasmid from DH5α was performed with the GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA) according to manufacturer instructions. Briefly, one colony from DH5α was grown in Luria broth (LB) and inoculated at 37°C overnight with agitation. The bacteria culture was centrifuged, the pellet was resuspended and the bacteria subsequently lysed. The suspension was later centrifuged and the pellet was washed. Plasmid DNA was bound in a column and then eluted after centrifugation. The purified plasmid DNA was stored at -20°C.

3.3.2 Transformation by electroporation

To prepare electrocompeotent bacterial cells, a colony of E7 grown on TSA plate was cultured in LB with agitation at 37°C and incubated overnight. The bacteria were then regrown in LB and incubated with agitation at 37°C for 4 hours. After incubation, the bacterial suspension was centrifuged for 15 minutes at 4000 x g at 4°C. The pellet was washed with ice-cold sterile ddH₂O.

Two microliters of 1 µg/µL plasmid DNA was mixed with 30 µL of bacterial suspension, transferred into a pre-chilled 0.1 cm gap cuvette, and electroporated (1.8 kV, 25 µF, 200 ohm, and ~5.0 msec) using Gene Pulser Xcell™ (Bio-Rad, USA). The bacterial cells were immediately resuspended with Super Optimal broth with Catabolite repression (SOC) and incubated with
agitation at 37°C for 60 minutes without gentamicin. After recovering the bacterial cells, the bacteria were plated on TSA plate in the presence of gentamicin.

### 3.4 Susceptibility testing before and after transformation
Susceptibility testing on E7 was performed before and after transformation, according to the recommendations by the Swedish Reference Group for Antibiotics ([www.srga.org](http://www.srga.org)). In addition, a minimal inhibitory concentration (MIC) of ceftibuten was determined on E7 as well. The MIC was determined as the lowest concentration at which ceftibuten prevented visible growth of the bacteria in LB containing phosphate buffered saline (PBS) incubated for 20 hours at 37°C.

### 3.5 Model of infection
Before infecting the 5637 cells, the pLMB449-introduced E7 (5*10⁶ CFU [colony-forming unit]) were cultured and recultured in LB supplemented with gentamicin (20 µg/mL) for two-three days under static condition at 37°C, then centrifuged at 4000 x g for 10 minutes. The pellet (3*10⁹ CFU) was resuspended in 0.3 mL PBS (1*10¹⁰ CFU/mL) and diluted to fit different multiplicities of infection (MOI).

The 5637 cells were seeded and cultured at ~2 x 10⁵ cells/mL in 24-well plates two-three days before infection with E7. At confluence, they were infected at different MOI with or without ceftibuten (480 ng/mL), and with or without the presence of gentamicin (10 µg/mL) for 6 or 24 hours. Cells incubated for 24 hours were washed with fresh cell culture media supplemented with 2% FBS 6 hours postinoculation.

For evaluation of the best model of infection to analyze with flow cytometry, cells were infected at MOI-0.1, MOI-1, MOI-5, and MOI-10 for 6 hours, and at MOI-0.01, MOI-0.1, MOI-1, MOI-0.5, and MOI-10 for 24 hours (Table 1.).

Prior to flow cytometric analysis, cells were seeded and cultured at ~2 x 10⁵ cells/mL in 6-well plates. Confluent, 5637 cells were infected at MOI-10 and MOI-1 for 6 hours with or without ceftibuten. Gentamicin was added to one well with MOI-10 with or without ceftibuten.
Table 1. Model of infection in 24-well plates for 6 hours and 24 hours in the presence and absence of ceftibuten and gentamicin.

<table>
<thead>
<tr>
<th>MOI*</th>
<th>6 hours</th>
<th>24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ceftibuten</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>0 (control)</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>0.01</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>0.1</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>0.5</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>1</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>5</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>10</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

* Multiplicity of infection

3.6 Microscopic evaluation
The 5637 cells were grown and cultured on plastic coverslip inserts (Sarstedt, Inc. Newton, USA) in 24-well plates. Infected cells were washed twice with PBS before mounted in PBS and then examined with an Olympus BX41 fluorescence microscope (Olympus, Segrate, Italy) with a mercury arc lamp Olympus U-RFL-T (Olympus, Segrate, Italy) as a light source. Images were captured using a Leica DFC420 camera (Leica, California, USA). 5637 cells cultured in 6-well plates were detached with 0.05% Trypsin-EDTA (Gibco, Paisley, UK) before the flow cytometric analysis. These cells were also examined with fluorescence microscope.

The following parameters were evaluated: the 5637 cells’ condition, the GFP-signal, the presence of adherent extracellular bacteria, the presence of filamentous bacteria after treatment with ceftibuten, the presence of intracellular bacteria, and the presence of IBCs.

3.7 Flow cytometric analysis
Cells were cultured in 6-well plates before infection with E7 of different MOI with or without ceftibuten, and with or without the presence of gentamicin. Post infection, the 5637 cells were rinsed twice with PBS, after which they were detached with trypsin at 37°C in a 5% CO₂ atmosphere. The cells were recovered with DMEM supplemented with 2% FBS. After detachment, the cells were centrifuged at 300 x g at 20°C for 5 minutes. Resuspension with 1 mL of PBS was then followed by determination of GFP-expression by using the Gallius™ (Beckman Coulter, Brea, CA, USA) flow cytometer with 488 nm laser and FL1 525/40 nm band-pass filter. The cells were gated and analyzed with Kaluza Flow Cytometry Analysis v1.3 (Beckman Coulter).
4. Ethical Considerations

The use of FBS obtained from fetal calves implies ethical considerations. However, it has been proven that the composition of growth factors and the lack of antibodies in FBS are essential for *in vitro* cell culturing. Therefore, the consideration is assessed to be of lower value than the expected beneficial outcome of the study.

E7 was isolated from a patient with urosepsis at Örebro University hospital, Sweden. The patient’s identity was anonymized prior to this study.

The human uroepithelial bladder cancer 5637 cells were originally isolated from a patient with urinary bladder carcinoma in 1974. The cells were purchased and grown as cell lines and cannot be linked to the original donor. Taken together, the experiments imply low ethical implications without the need for an ethical application.

5. Results

5.1 Visualization of GFP-labeled E7

To evaluate whether E7 received the pLMB449-plasmid or not after transformation, the bacteria were visualized with fluorescence microscopy after transformation. E7 were also treated with a sub-MIC concentration (480 ng/mL) of ceftibuten to verify that it retained its morphological plasticity in the presence of ineffective antibiotic. A strong GFP-signal was observed in both coliform and filamentous E7 (Fig. 2.).

![Fig. 2. Fluorescence microscopy of GFP-labeled E7. Coliform (A [cultured in the presence of gentamicin]) and filamentous (B [cultured in the presence of gentamicin and ceftibuten]) E7 expressing GFP indicating that the pLMB449-plasmid has been successfully transformed.](image-url)
5.2 Antibiotic resistance patterns of E7 before and after transformation

Susceptibility testing on E7 before and after transformation was performed to verify possible changes in resistance patterns of E7 after introducing the pLMB449-plasmid. The susceptibility testing shows that E7 kept its previous pattern of resistance and also gained resistance to gentamicin (Table 2.). In addition, a MIC of ceftibuten was determined on E7 as well and was found to be 3840 ng/mL.

Table 2. Resistance patterns of E7 before and after pLMB449-transformation.

<table>
<thead>
<tr>
<th>Antibacterial agent</th>
<th>Before transformation (S*/R**)</th>
<th>After transformation (S/R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Trimethoprim/sulfamethoxazole</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Meropenem</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Ceftibuten***</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Mecillinam</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Amoxicillin clavulanic acid</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

*S: Susceptible  
**R: Resistant  
***MIC of ceftibuten: 3840 ng/mL

5.3 Microscopic evaluation of the different infection models

A microscopic evaluation was performed with respect to the following parameters: the 5637 cells’ condition, the GFP-signal, the presence of adherent extracellular bacteria, the presence of filamentous bacteria after treatment with ceftibuten, the presence of intracellular bacteria (loosely packed), and the presence of IBC. These parameters were evaluated in both attached and detached 5637 cells.
5.3.1 Attached uroepithelial cells

Generally, there were some difficulties determining whether the bacterial cells were inside or resided atop the uroepithelial cells. Some IBCs were very distinctive, while other IBCs had a lesser characteristic appearance. Bacterial cells showed a filamentous morphology after treatment with ceftibuten, whereas bacterial cells not treated with ceftibuten were still coliform after 6 hours of inoculation. The GFP-signal was greater in the presence of gentamicin compared to in the absence of this selective agent.

Cells infected at MOI 0.1 with and without ceftibuten for 6 hours were still confluent (comparable to the control wells). Several bacterial communities were observed but whether they were intracellular or extracellular was in some cases difficult to determine (Fig. 3; A-C). Regarding adherent extracellular bacterial cells, there were more adhesive rod-shaped bacteria in the absence of ceftibuten compared to that of filamentous bacteria in the presence of ceftibuten.

Infection models at MOI 1 and 5 for 6 hours resulted in similar observations. However, the cells were not fully confluent, uncovered patches were observed which indicated that the cells were more damaged compared to MOI 0.1. When ceftibuten was added, the cells were less damaged than when ceftibuten was excluded. The number of extracellular bacterial cells was higher in MOI 1 and 5 compared to MOI 0.1, and there were more adhesive bacteria in MOI 1 and 5 (Fig. 3; D). Less adherent bacteria were observed in wells containing ceftibuten (Fig. 3; E). Many bacterial communities were found both in the presence and absence of ceftibuten, but only a minority of them could with certainty be classified as IBCs.

5637 cells infected at MOI 10 for 6 hours were even less confluent than cells infected at lower MOI. When ceftibuten was added, numerous long filaments were observed. After washing the cells with PBS twice before microscopic evaluation, the filamentous bacteria were starting to revert to its’ original rod-shaped morphology (Fig. 3; F). Bacterial adhesion was more significant in the absence of ceftibuten (Fig. 3; G and H). More bacterial clusters were observed in the absence of ceftibuten than when ceftibuten was added.

Moreover, some uroepithelial cells harbored possible intracellular filamentous bacterial cells (Fig. 3; I and J).

Cells infected at MOI 0.01 for 24 hours in wells without ceftibuten-supplementation were less confluent in comparison to control wells and wells containing ceftibuten. Few bacterial cells, both intracellular and extracellular were found. In ceftibuten-supplemented wells, few filaments were
observed after 24 hours of incubation. The bacterial cells had reverted to their original rod-shaped morphology.

Twenty-four hours of infection at MOI 0.1 and 0.5 with and without ceftibuten resulted in comparable observations. The initially confluent uroepithelial cells were not confluent 24 hours postinfection. Uncovered areas were noticed. Moreover, the filamentous morphology of the bacteria did not exist after ceftibuten-depletion. The rod-shaped bacteria were strongly attached to the bladder cells, which was more apparent when ceftibuten was excluded (Fig. 4; A and B). Bacterial communities were observed as well, but the majority of these were deemed to be extracellular.

After infection at MOI 1 for 24 hours, the 5637 cells were more viable in the presence of ceftibuten compared to when ceftibuten was excluded. More bacteria were attached to the bladder cells in ceftibuten-negative wells (Fig. 4; C). Several bacterial clusters were observed in both lines of experiments, but the validity of their intracellular position remains uncertain.

Lastly, cells infected at MOI 10 for 24 hours with and without ceftibuten, respectively, were not viable and it was difficult to evaluate the different parameters in these infection models.
Fig. 3. Fluorescence microscopy of 6 hours infection models. A minority of the bacterial communities were distinctive intracellular (A [MOI 0.1 with ceftibuten]) showing clear vesicular structure in visible light (B [MOI 0.1 with ceftibuten]). The majority of the biofilm-like collections of bacteria were either indistinguishable intracellular (C, arrow [MOI 0.1 with gentamicin]) or extracellular (C, arrowhead). More rod-shaped bacterial cells were adhered to uroepithelial cells in the absence of ceftibuten (D [MOI 1] and G [MOI 10 with gentamicin]) compared to when ceftibuten was added where filamentous bacteria were less adhesive (E [MOI 5 with ceftibuten and gentamicin] and H [MOI 10 with ceftibuten and gentamicin]). Filamentous E7 began reverting to their original rod-shape (F [MOI 10 with ceftibuten]) after rinsing with PBS. Rare observations revealed the fact that some filamentous bacteria became intracellular (I [MOI 1 with ceftibuten and gentamicin] and J [MOI 1 with ceftibuten and gentamicin]).
Fig. 4. Fluorescence microscopy of 24 hours infection models. When ceftibuten was excluded, the rod-shaped E7 showed stronger and more significant adhesion to the bladder cells (A [MOI 0.1 with gentamicin] and C [MOI 1]) than ceftibuten-treated, previously filamentous bacteria did (B [MOI 0.1 with ceftibuten]). Some distinct IBCs were also observed after 24 hours of infection (D-F [MOI 1]).

5.3.2 Detached cells with trypsin

Based on the above reported results, the 6 hours models of infection were evaluated to be the most appropriate to analyze with flow cytometry. Before flow cytometric analysis, the cells were detached with trypsin and a sample of each model was evaluated by fluorescence microscopy.

After detachment of the uroepithelial cells, individual bacterial cells were observed rather than biofilm-like bacterial collections. Thus, it became easier to evaluate the position of the bacteria, intracellular or extracellular. However, the observations made in attached-cell models were consistent with the observations made after detachment of the 5637 cells. Ceftibuten-treated (filamentous) bacteria were less adhesive (Fig. 5; A) and the urothelial cells were more viable compared to ceftibuten-negative wells (Fig. 5; B). In addition, the GFP-signal was stronger in the presence of gentamicin.

The amount of filamentous cells after cetibuten-supplementation was less after detachment with trypsin. Moreover, after detachment, less numerous extracellular bacteria were observed in both ceftibuten-positive and negative samples. However, most of the bacterial cells were found to be extracellular, surrounding the bladder cells (Fig. 5; C), but intracellular bacteria were also observed, especially in the absence of ceftibuten (Fig. 5; D). When it comes to ceftibuten-treated bacteria, the
intracellular bacteria were coliform and not filamentous (Fig. 5; E). Furthermore, some of the intracellular bacteria seemed to have lost their GFP-expression.

**Fig. 5.** Fluorescence microscopy of 6 hours infection models after detachment of the uroepithelial cells with trypsin. Ceftibuten-treated, filamentous bacteria showed less significant adhesion to the bladder cells (A [MOI 10 with ceftibuten and gentamicin]) than ceftibuten-depleted, coliform E7 did (B [MOI 10 with gentamicin]). Most of the bacterial cells were found to have an extracellular position, binding to the 5637 cells (C [MOI 10]). Additionally, rod-shaped bacteria were observed within the bladder cells, both in the absence (D [MOI 10]) and presence of ceftibuten (E [MOI 10 with ceftibuten]).

5.4 Flow cytometric analysis of adherent and intracellular bacteria

Exclusion of non-viable cells from the analysis was accomplished by using forward scatter (FS) gating (Fig. 6; A). GFP-positive and negative cells were distinguished on the basis of background fluorescence of 5637 cells that were not infected and the cut-off level was set between two distinct populations of cells (Fig. 6; B). 5637 cells were gated according to their GFP-signal (Fig. 6; B, C and D) i.e. whether the bladder cells were GFP-positive or negative. However, it was difficult to determine whether the GFP-expressing bacteria were intracellular or adherent to the bladder cells without secondary staining of extracellular bacteria.

The GFP-expression in 5637 cells infected with E7 for 6 hours was higher in ceftibuten-negative wells than in ceftibuten-positive wells (Table 3.). The GFP-signal was stronger when gentamicin was added, especially in ceftibuten-treated uroepithelial cells (Table 3.).
Fig. 6. Representative experiment (MOI 10 with gentamicin) showing gating strategies for analyzing the GFP-expression in E7-infected 5637 cells. By using forward scatter (FS) gating cellular debris was excluded from the analysis (A). The histogram (B) shows the GFP-expression in 5637 cells divided into two separate subpopulations with GFP-positive and GFP-negative cells. The GFP/SS-gated plot (C) was used to evaluate the source of the GFP-signal (i.e. from intracellular or adherent E7). In order to determine whether the uroepithelial cells were GFP-positive or negative, the GFP/FS-gated dot plot (D) was used.
Table 3. The GFP-expression in 5637 cells infected at different MOI for 6 hours with or without ceftibuten and gentamicin, respectively.

<table>
<thead>
<tr>
<th>MOI*</th>
<th>GFP**-positive (%)</th>
<th>GFP-negative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>1.4</td>
<td>98.6</td>
</tr>
<tr>
<td>1</td>
<td>92.7</td>
<td>7.3</td>
</tr>
<tr>
<td>1+Ceftibuten</td>
<td>82.5</td>
<td>17.5</td>
</tr>
<tr>
<td>10</td>
<td>93.2</td>
<td>6.8</td>
</tr>
<tr>
<td>10+Ceftibuten</td>
<td>68.9</td>
<td>31.1</td>
</tr>
<tr>
<td>10+ Gentamicin</td>
<td>93.5</td>
<td>6.5</td>
</tr>
<tr>
<td>10+Ceftibuten + Gentamicin</td>
<td>86.3</td>
<td>13.7</td>
</tr>
</tbody>
</table>

*Multiplicity of infection  
**Green fluorescent protein

6. Discussion

The main purpose of this study was to set up and optimize an in vitro model of infection that enabled the detection of intracellular ESBL-producing UPEC by GFP. After introduction of the GFP-expressing pLMB449-plasmid into E7 (Fig. 2.), several infection models could be set up using 5637 bladder cells for an in vitro infection. Models of infection at different MOI in the presence and absence of ceftibuten and gentamicin, respectively, were tested for 6 hours and 24 hours of inoculation, respectively (Table 1.).

Microscopic evaluation of attached urothelial cells infected for 6 hours showed that bacterial adhesion was less significant in the presence of ceftibuten (Fig. 3.) and that the 5637 cells were more viable when ceftibuten was added. This suggests that filamentous bacteria may be less virulent compared to rod-shaped bacteria. Moreover, infection models for 24 hours resulted in comparable observations to those obtained from 6 hours of infection (Fig. 4.), suggesting that previously filamentous E.coli have decreased virulence despite reversion (i.e. reverting from filamentous morphology to rod-shaped morphology).

Distinctive IBCs were found in cells infected for both 6 hours and 24 hours (Fig. 3. and 4.), but the majority of the biofilm-like bacterial collections observed were indistinctive intracellular or extracellular (Fig. 3.). These extracellular biofilms might have been formed extracellularly, or formed as a result of lysed, former IBC-containing bladder cells.
Another interesting finding was that cells infected at MOI 0.01 for 24 hours were less confluent than cells infected at MOI 0.1 for the same amount of time. This may be associated to the ability of *E. coli* to induce proliferation in host cells [25]. Thus, uroepithelial cells infected at low MOI could exhibit a lower proliferative host response.

Based on the aforementioned results the experiments preceded with an infection model at MOI 10 and 1 with and without the presence of ceftibuten at 6 hours of infection. Cells infected in these models were detached with trypsin and evaluated with fluorescence microscope and analyzed with flow cytometry. Microscopic observations of these cells revealed individual intracellular bacterial cells rather than biofilm-like IBCs (Fig. 5.). Detachment with trypsin could have damaged the vulnerable IBC-harboring 5637 cells leading to this observation. Difficulties in induction of IBCs in 5637 cells may be due to the higher density of β-actin in these cells compared to differentiated uroepithelial cells [26]. Furthermore, 5637 cells lack the expression of uroplakin Ia [27,28], and express only α3β1 integrins as binding molecules for UPEC [29]. Thus, it is more difficult for E7 to form IBCs in the bladder cells used in this study.

In addition, studies have shown that ESBL-producing UPEC isolates are less virulent than classical UPEC isolates [30-32]. Due to incompatibility, virulence genes encoded by plasmids can be outcompeted by resistance genes encoded by other plasmids [32]. Thereby, E7 used in this study might be less capable of establishing IBCs, especially in the more infection resistant 5637 cells.

After cellular detachment, less adherent bacteria were observed (Fig. 5.). This could be explained by the effect that trypsin has on protein linkages between the bacteria and the bladder cells. Intracellular bacterial cells in the presence of ceftibuten were seen to be coliform (Fig. 5.). Ceftibuten is an extracellular agent and thus defilamentation of the bacterial cells occur in the ceftibuten-depleted intracellular milieu.

The detached 5637 cells were analyzed using flow cytometry. Data from this analysis (Table 3.) reveals that bladder cells infected with E7 in the absence of ceftibuten show a greater GFP-expression. This could be explained by the fact that filamentous bacteria (ceftibuten-treated) are less virulent than rod-shaped UPEC. Ceftibuten constitutes a selective pressure on resistance-plasmids in E7 leading to changes in virulence characteristics of the bacteria, a phenomena referred to as biological fitness cost [24]. A reduced growth rate is an example of biological fitness cost [24] and may in this case lead to decreased pathogen pressure on the bladder cells. Thus, coliform bacteria may be more virulent (i.e. more adhesive) than filamentous bacteria.
and more likely to invade the uroepithelial cells. Additionally, the GFP-signal was stronger when gentamicin was added, especially in ceftibuten-treated uroepithelial cells (Table 3.). Gentamicin selects the GFP-expressing pLMB449-plasmid in E7 and thus leads the absence of gentamicin to reduced GFP-expression in extracellularly positioned bacteria.

It is important to emphasize that the GFP-signals analyzed by the flow cytometer is not emerging from intracellular bacteria alone, but also from extracellularly adherent bacteria. After detachment, as described above, most of the observed intracellular bacteria were individually separated and thereby leading to no remarkable changes in the 5637 cells’ complexity, distinguished with side scatter (SS), as IBCs would have shown. Consequently, it was difficult to analyze the GFP/SS gated plot giving rise to the inability to separate IBC carrying 5637 cells. On the other hand, gentamicin does not act intracellularly, whereby pLMB449-plasmid selection ceases. This might lead to the presence of intracellular GFP-silent bacteria giving rise to false negative signal.

There are some limitations in this study. Firstly, it was difficult to evaluate the bladder cells and the GFP-expressing E7 in the attached state with regular fluorescence microscope. Improvement can be made by examining the cells in the detached state and/or using confocal fluorescence microscopy to obtain three-dimensional images. Moreover, the microscopic evaluation of the GFP-expression was based on subjective evaluation and is therefore not standardized. When it comes to flow cytometric analysis, the GFP-signal derived from extracellularly adherent E7 could not be excluded with the GFP/SS-gate. This can instead be accomplished by coating the extracellular E7 with antibodies (e.g. against type 1 pili) and then gate against this secondary signal in flow cytometry. Unfortunately, the flow cytometric analysis of the GFP-expression was performed only once due to limited time. Thus, the results obtained from this analysis are not statistically analyzed.

There are some mentionable strengths in this study. One strength is that cells incubated for 24 hours were washed with fresh DMEM to mimic in vivo infections where urine rinses the bladder from extracellular, loosely adherent bacteria. Furthermore, the use of GFP-expression enables tracking of living bacterial cells and thereby, dead intracellular bacteria will be excluded and will not be mistaken as viable intracellular bacteria. Lastly, serial culturing of E7 in LB for two-three days was done to achieve optimal type 1 pili-expression that has been shown to enhance the virulence of UPEC [33].
7. Conclusion
The transformation of pLMB449-plasmid into E7 was successful and the bacteria kept its previous pattern of resistance, gained resistance to gentamicin, and retrained its morphological plasticity in the presence of ineffective antibiotic. The results from in vitro infection models demonstrate that the 6 hours models of infection are convenient to induce intracellular bacteria which then can be analyzed using flow cytometry to obtain quantitative results. However, further optimization is needed to achieve optimal models of infection.

8. Further research
Further optimization is needed to achieve optimal models of infection in order to study the intracellular pathogenic pathway of UPEC isolates in UTIs, in particular ESBL-producing UPEC. The microscopic evaluation should be performed using confocal microscope to obtain easily evaluated observations. Moreover, before analyzing the cells with flow cytometry, GFP-signals from extracellularly adherent bacterial cells should be attenuated using antibodies against UPEC (e.g. anti-type pili antibodies). After optimization of infection models and analysis methods on 5637 cells, the optimized parameters will be applied on human bladder epithelial primary cells (HBEP) to more resemble an in vivo infection. Subsequently, GFP-positive bladder cells (i.e. invaded by E7) and GFP-negative cells will be sorted by fluorescence-activated cell sorting (FACS). The last step in this research will consist of running mRNA microarray analysis of these two separate subpopulations of cells to evaluate differences in mRNA-expression in invaded bladder cells and cells that elude bacterial invasion.

9. Acknowledgements
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10. References


