Detection of intracellular bacterial communities in a child with *Escherichia coli* recurrent urinary tract infections

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This work verifies that *E. coli* can be intracellular in cases of severe recurrent urinary tract infections.

**Keywords**

*Escherichia coli*; UTI; intracellular bacterial communities; UPEC; children.

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**Abstract**

The formation of intracellular bacterial communities (IBC) has been proposed as a new pathogenic model for urinary tract infections. Scarce reports describe this phenomenon in humans. We describe the presence of IBC in uroepithelial cells of a child with recurrent urinary infections. Urine specimen was collected from a child with *Escherichia coli* UTI and analyzed by light and confocal laser scanning microscopy (CLSM). The capability of this strain to produce intracellular infection in bladder tissue was confirmed in mice models. *Escherichia coli* phylogenetic group, presence of virulence factors genes, and its multiple locus sequence type were determined. CLSM showed large collections of morphologically coccoid and rod bacteria in eukaryotic cells cytoplasm, even seemingly protruding from the cells. *Escherichia coli* EC7U, ST3626, harbored type 1, P, and S/F1C fimbriae and K1 capsule genes. In this report, we confirm the presence of IBC in children with UTI, as it has been described before in women.

**Urinary tract infections** (UTIs) generate a major problem in children, being *Escherichia coli* responsible for 80–90% of UTIs (Habib, 2012).

Approximately 1–8% of children between 1 month and 11 years have experienced at least one UTI which are usually conceived as a marker of urinary track morphological or functional abnormalities (Le Saux et al., 2000).

Recurrent UTIs (RUTIs) occur in 20–40% of children along the following 12 months from the first infection episode, being vesicourinary reflux (VUR) the most important risk factor (Le Saux et al., 2000; Garin et al., 2006; Conway et al., 2007).

Taking into account that VUR only explains 13–40% of RUTIs, additional explanations are needed (Nuutinen & Uhari, 2001; Conway et al., 2007).

Recently, new insights into the pathogenesis of uropathogenic *E. coli* (UPEC) have emerged. UPEC can invade the urothelium persisting as individual quiescent intracellular reservoirs or forming large biofilm-like inclusions known as intracellular bacterial communities (IBC) (Anderson et al., 2003; Mysorekar & Hultgren, 2006; Blango & Mulvey, 2010). Bacteria can emerge from these reservoirs, usually adopting a filamentous morphology. Filamentous bacteria could reinvoke urothelial cells initiating a new IBC formation cycle (Anderson et al., 2003; Mysorekar & Hultgren, 2006; Rosen et al., 2007).

IBC is proposed as a cause of RUTI, but no clinical studies have confirmed this hypothesis yet (Blango & Mulvey, 2010).

To the best of our knowledge, only Rosen et al. have described IBC in exfoliated bladder cells in urine from women with cystitis but no data of IBC formation in children have been reported yet (Rosen et al., 2007).

In this report, we described the presence of IBC in uroepithelial cells from urine of a child with UTI and performed the characterization of the infecting UPEC strain.
The urine specimen was collected in May 2012 from a 9-year-old girl with RUTIs caused by *E. coli*, who had no underlying morphological or functional urinary tract abnormalities, assisted at the emergency department of a teaching hospital because of cystitis. The urinary analysis showed leukocytes, nitrites, and red cells. Uroculture yielded more than 10⁶ CFU mL⁻¹ of *E. coli*. The recovered strain, named Ec7U was susceptible to all antibiotic tested [determined by Vitek 2 Compact system (bioMérieux, Marcy l’Étoile, France), and interpreted according to CLSI guidelines (CLSI, 2009)].

Phylogenetic group and the presence of 25 virulence factors genes were determined by multiplex-PCR according to previous reports (Clermont et al., 2000; Johnson et al., 2003).

Ec7U belonged to the phylogenetic group B2 and was positive for *limH* (type 1 pili), *papG* allele 3 (type P pili), *sfa/focDE* (pili S and F1), *KpsMTII* K1, *fyuA* (siderophore), and PAI (pathogenicity island) virulence factors gene.

Multiple locus sequence typing (MLST) was achieved as previously described by gene amplification and sequencing of seven housekeeping genes (*adh, furC, gyrB, icd, mdh, purA, and recA*) according to the *E. coli* MLST web site (http://mlst.ucc.ie/mlst/dbs/Ecoli).

Results indicated that *E. coli* Ec7U belongs to a new sequence type 3626 (ST3626; allelic profile, 4, 52, 10, 14, 2921 strain that forms biofilm was used as a positive control (Schlapp et al., 2013), and interpreted according to CLSI guidelines (CLSI, 2009).

Acquisition and processing of 3D image stacks were performed as described before (Schlapp et al., 2011) using 350/460, 488/520, and 543/565 excitation/emission wavelength. Acquisition step size was of 0.3 μm in the z-axis and 1024 × 1024 pixels in xy-plane with a pixel size of 70 nm. 3D Image stack was deconvolved with Huygens Scripting Software and were reconstructed using Volocity 3D Image Analysis Software (Perkin-Elmer).

CLSM and 3D Image reconstruction revealed communities of *E. coli* within and protruding from uroepithelial cells (Fig. 1). A large collection of morphologically coccoid and rod bacteria was observed in the cytoplasm of the cells that appeared limited by the WGA staining (blue) that marked the eukaryotic cell membrane. Also, UPIII (red) was detectable at the cells cytoplasm and certain membrane portions. Z planes revealed also that the membrane was disrupted indicating a bacterial dissemination step of IBC. Filamentous bacteria were also evidenced. All these results were consistent with IBC formation (Rosen et al., 2007).

We confirmed Ec7U capability of infection in bladder tissue on an experimental mouse model of ascending UTI (Zunino et al., 1994). The protocol consisted in the administration of 2 × 10⁸ CFU in 50 μL of PBS per animal. Female mice were anesthetized with a mix of xylazine (10 mg kg⁻¹) and ketamine (50 mg kg⁻¹), and bladders were voided by gentle massage of the abdomen before challenge. A soft polyethylene catheter was inserted through the urethra, and the bacterial suspension was slowly introduced into the bladder. Three days after infection, mice were sacrificed, bladders were aseptically removed and prepared for histological sectioning for immunofluorescence stain for confocal microscopy visualization. The immunofluorescence protocol stain was the same as described above. Image analysis and 3D image reconstruction showed intracellular bacteria surrounding the nucleus, confirming the presence of intracellular bacteria in bladder tissue (Fig. 1). All protocols with animals were evaluated and approved by the Committee of animal experimentation (CEUA) at IIBCE. Mice were kept with food and water *ad libitum* in the IIBCE facility during the entire protocol.

The phylogenetic group B2 and the presence of different virulence factors, such as adhesins, capsule, and syndero-phores, defined Ec7U as a real uropathogen. Ec7U was positive for type 1, P and S/F1C fimbriae, and K1 capsule, which have been involved in IBC formation (Dhakal et al., 2008; Anderson et al., 2010).

The classical mechanism of acute and recurrent UTI proposed the ascension of bacteria from the gut microbiota to the vagina and then to the bladder. However, this does...
not explain why up to 70% of RUTIs are caused by *E. coli* identical to the original infecting strain (Garofalo et al., 2007; Koljalg et al., 2009) IBC and the eventual emergence of bacteria from these quiescent intracellular reservoirs, rein- 
vading new urothelial cells, may explain a significant 
percentage of RUTIs.

In this report, we detect IBC formation in a young girl with 
RUTIs due to *E. coli*. The quiescent status of intracellular 
bacteria in IBC makes them less detectable by the host 
immune system and also less susceptible to antibiotics 
treatments, even though *in vitro* susceptibility to many 
antibiotics. Scarce studies have evaluated antibiotic efficacy 
against intracellular *E. coli*. Blango et al. proposed spar-
floxacin–fosfomycin association as a possible option for IBC 
eradication (Blango & Mulvey, 2010).

The evidence of IBC presence in urine of children with UTI 
would justify the reconsideration of the empirical UTI 
treatment, at least in the cases of RUTIs, being fluoroquino-
lones a possible option. Despite quinolones use has 
been restricted in pediatrics, a low resistance level mediated 
by transferable genes such as *qnr* have been described 
(Garcia-Fulgueiras et al., 2011).

Based on this study, we are developing a project in order 
to determine the prevalence of IBC in children and its 
relationship with recurrent UTIs.

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