Pathogens and Disease ISSN 2049-632X

SHORT COMMUNICATION

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

Luciana Robino[1], Paola Scavone[2], Lucia Araujo[1], Gabriela Algorta[1,3], Pablo Zunino[2] & Rafael Vignoli[1]

1 Departamento de Bacteriología y Virología, Instituto de Higiene, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay
2 Departamento de Microbiología, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay
3 Laboratorio Central del Hospital Pereira Rossell-Ministerio de Salud Pública, Montevideo, Uruguay

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.

Correspondence
Rafael Vignoli, Departamento de Bacteriología y Virología, Instituto de Higiene, Facultad de Medicina, Universidad de la República. Alfredo Navarro 3051, CP 11600, Montevideo, Uruguay.
Tel.: +0598 24875795
fax: +0598 24875795
e-mail: rvignoli@higiene.edu.uy

Received 3 May 2013; revised 15 May 2013; accepted 16 May 2013. Final version published online 26 June 2013.

doi:10.1111/2049-632X.12047

Editor: Ake Forsberg

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 tract 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 reinvade 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.

Pathogens and Disease
FEMS

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 urinalysis showed leukocytes, nitrites, and red cells. Uroculture yielded more than 10^5 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 *fimH* (type 1 pili), *papG* allele 3 (type P pili), *sfa/focDE* (pili S and F1), *KpsMT*II *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 (*adk, fumC, 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, 17, 25, 17).

Escherichia coli Ec7U ability to form biofilm was analyzed according to the protocol described by Sheikh and modified by Perelmuter *et al.* (Sheikh *et al.*, 2001; Perelmuter *et al.*, 2008). The characterized *Proteus mirabilis* 2921 strain that forms biofilm was used as a positive control (Schlapp *et al.*, 2011) and medium without bacteria was used as a negative control. The absorbance was read at 545 nm in a spectrophotometer (Varioskan). The assay was performed in duplicates in two independent experiments, and the averages and standard deviations were calculated.

The $OD_{545\ nm}$ value corresponding to *E. coli* Ec7U biofilm was 0.201 ± 0.15 while the *P. mirabilis* biofilm forming strain showed an $OD_{545\ nm}$ value of 1.007 ± 0.136. The difference between both $OD_{545\ nm}$ values was significant ($P = 0.046$, Duncan Test). Comparison of these results allowed us to conclude that under the conditions used to perform this semiquantitative assay *E. coli* Ec7U was not able to form a strong biofilm.

Urine specimen was cytocentrifugated, and slides for light microscopy and CLSM were prepared as previously described (Rosen *et al.*, 2007). Light microscopy analysis revealed large and dark staining exfoliated epithelial cells, with attached and potentially intracellular bacteria. Also, long filamentous bacteria were seen, probably emerging from uroepithelial cells (Fig. 1a–c).

Immunofluorescence staining was performed using a specific *E. coli* antibody coupled to FitC (Abcam), Uroplakine III (UPIII, Santa Cruz biotechnology), Alexa Fluor(R) 568 donkey anti-goat IgG- and Alexa Fluor (R) 350-WGA (Molecular Probes). Slides were fixed with 4% of PFA, 15 min. After washing with PBS, WGA (5.0 $\mu g\ mL^{-1}$) and UPIII (1/50) staining was performed during 15 min and then cells permeabilization was performed. After 15 min, slides were washed and incubated with *E. coli* antibody (1/50) and Uroplakine III (1/50) 1 h, RT. Then, the samples were washed with PBS and incubated with donkey anti-goat antibody (1/400) and WGA (5.0 $\mu g\ mL^{-1}$) for 30 min. Once staining was over the slides were mounted with 10 μL of citifluor (r).

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 (PerkinElmer).

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 2×10^8 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 syderophores, 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

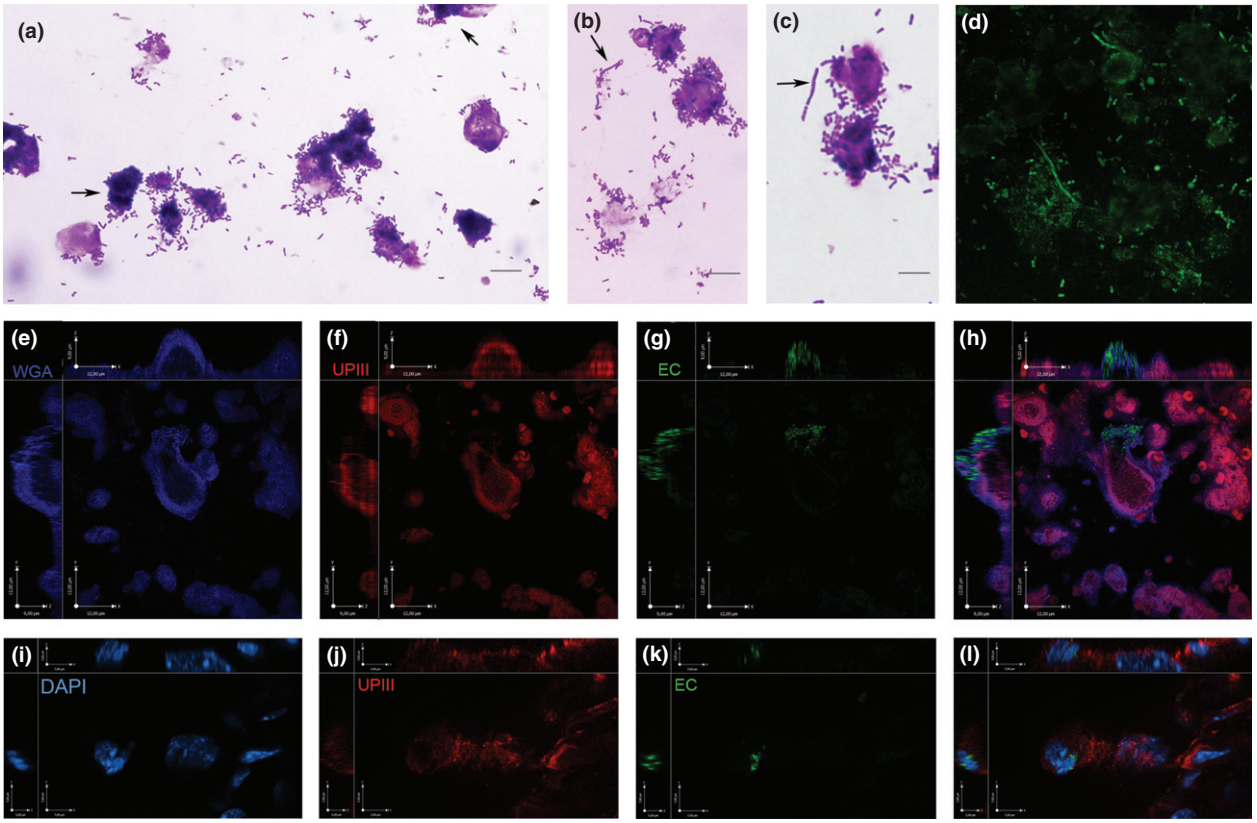

Fig. 1 IBC detection in urine of a child with UTI. (a–c) Light microscopy of exfoliated uroepithelial cells after Wright Giemsa staining revealed dark stained cells surrounded by bacteria (arrows) and the typical long filamentous (arrows). Scale bars represent 10 and 5 μm in (a) and (b, c), respectively. (d) Long filamentous *Escherichia coli* detected by immunofluorescence. (e–h) CSLM images of exfoliated uroepithelial cells stained with WGA (blue, e), UPIII (red, f), and *E. coli* (green, g). 3D stack images revealed a cell with its membrane partially disrupted and bacteria within and protruding the cell (h). (i, j) Bladder section of a mice infected with EcU7, the tissue section was stained with DAPI for DNA detection (blue, i), UPIII (red, j), *E. coli* (green, k). 3D stack images of the three channels revealed a small group of intracellular bacteria in a perinuclear localization (l).

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, reinvading 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 sparfloxacin–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 fluoroquinolones 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.

Acknowledgements

This work was partially supported by grants from ANII (Agencia Nacional de Investigación e Innovacion) to LR and CSIC (Comisión Sectorial de Investigación Científica, Uruguay) to RV. The authors declare no conflict of interest.

References

Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J & Hultgren SJ (2003) Intracellular bacterial biofilm-like pods in urinary tract infections. *Science* 301: 105–107.

Anderson GG, Goller CC, Justice S, Hultgren SJ & Seed PC (2010) Polysaccharide capsule and sialic acid-mediated regulation promote biofilm-like intracellular bacterial communities during cystitis. *Infect Immun* 78: 963–975.

Blango MG & Mulvey MA (2010) Persistence of uropathogenic *Escherichia coli* in the face of multiple antibiotics. *Antimicrob Agents Chemother* 54: 1855–1863.

Clermont O, Bonacorsi S & Bingen E (2000) Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* 66: 4555–4558.

CLSI (2009) Performance Standards for Antimicrobial Susceptibility Testing; Nineteenth Informational Supplement Vol. 29. Clinical and Laboratory Standards Institute, Wayne, PA.

Conway PH, Cnaan A, Zaoutis T, Henry BV, Grundmeier RW & Keren R (2007) Recurrent urinary tract infections in children: Risk factors and association with prophylactic antimicrobials. *JAMA* 298: 179–186.

Dhakal BK, Kulesus RR & Mulvey MA (2008) Mechanisms and consequences of bladder cell invasion by uropathogenic *Escherichia coli*. *Eur J Clin Invest* 38(Suppl 2): 2–11.

Garcia-Fulgueiras V, Bado I, Mota MI *et al.* (2011) Extended-spectrum β-lactamases and plasmid-mediated quinolone resistance in enterobacterial clinical isolates in the paediatric hospital of Uruguay. *J Antimicrob Chemother* 66: 1725–1729.

Garin EH, Olavarria F, Garcia Nieto V, Valenciano B, Campos A & Young L (2006) Clinical significance of primary vesicoureteral reflux and urinary antibiotic prophylaxis after acute pyelonephritis: A multicenter, randomized, controlled study. *Pediatrics* 117: 626–632.

Garofalo CK, Hooton TM, Martin SM, Stamm WE, Palermo JJ, Gordon JI & Hultgren SJ (2007) *Escherichia coli* from urine of female patients with urinary tract infections is competent for intracellular bacterial community formation. *Infect Immun* 75: 52–60.

Habib S (2012) Highlights for Management of a Child with a Urinary Tract Infection. *Int J Pediatr* 2012: 6.

Johnson JR, Kuskowski MA, Owens K, Gajewski A & Winokur PL (2003) Phylogenetic origin and virulence genotype in relation to resistance to fluoroquinolones and/or extended-spectrum cephalosporins and cephamycins among *Escherichia coli* isolates from animals and humans. *J Infect Dis* 188: 759–768.

Koljalg S, Truusalu K, Vainumae I, Stsepetova J, Sepp E & Mikelsaar M (2009) Persistence of *Escherichia coli* clones and phenotypic and genotypic antibiotic resistance in recurrent urinary tract infections in childhood. *J Clin Microbiol* 47: 99–105.

Le Saux N, Pham B & Moher D (2000) Evaluating the benefits of antimicrobial prophylaxis to prevent urinary tract infections in children: a systematic review. *CMAJ* 163: 523–529.

Mysorekar IU & Hultgren SJ (2006) Mechanisms of uropathogenic *Escherichia coli* persistence and eradication from the urinary tract. *P Natl Acad Sci USA* 103: 14170–14175.

Nuutinen M & Uhari M (2001) Recurrence and follow-up after urinary tract infection under the age of 1 year. *Pediatr Nephrol* 16: 69–72.

Perelmuter K, Fraga M & Zunino P (2008) *In vitro* activity of potential probiotic *Lactobacillus murinus* isolated from the dog. *J Appl Microbiol* 104: 1718–1725.

Rosen DA, Hooton TM, Stamm WE, Humphrey PA & Hultgren SJ (2007) Detection of intracellular bacterial communities in human urinary tract infection. *PLoS Med* 4: e329.

Schlapp G, Scavone P, Zunino P & Hartel S (2011) Development of 3D architecture of uropathogenic *Proteus mirabilis* batch culture biofilms-A quantitative confocal microscopy approach. *J Microbiol Methods* 87: 234–240.

Sheikh J, Hicks S, Dall'Agnol M, Phillips AD & Nataro JP (2001) Roles for Fis and YafK in biofilm formation by enteroaggregative *Escherichia coli*. *Mol Microbiol* 41: 983–997.

Zunino P, Piccini C & Legnani-Fajardo C (1994) Flagellate and non-flagellate *Proteus mirabilis* in the development of experimental urinary tract infection. *Microb Pathog* 16: 379–385.