Intracellular Bacteria in the Pathogenesis of *Escherichia coli* Urinary Tract Infection in Children

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Background. Uropathogenic *Escherichia coli* (UPEC) is the most common agent of urinary tract infection (UTI). The classic model of pathogenesis proposes the ascent of UPEC by the urethra and external adherence to the urothelium. Recently, the ability of UPEC to invade urothelial cells and to form intracellular bacterial communities (IBCs) has been described.

Methods. The objective of the present study was to determine the presence of intracellular bacteria (IB) in children with UTI caused by *E. coli* and to characterize its virulence attributes and its relation with clinical outcomes. One hundred thirty-three children with *E. coli* UTI who attended a reference children's hospital between June and November 2012 were included. Urine samples were analyzed by optical and confocal microscopy looking for exfoliated urothelial cells with IB. Phylogenetic group and 24 virulence factors of UPEC were determined using multiplex polymerase chain reaction. Medical records were analyzed.

Results. The presence of IB was detected in 49 of 133 (36.8%) samples by confocal microscopy, in 30 cases as IBC, and in 19 as isolated intracellular bacteria (IIB). Only 50% of these cases could be detected by light microscopy. Seventy-four medical records were analyzed, 34 with IBC/IIB, 40 without IB. Any virulence gene was associated with IBC/IIB. The presence of IBC/IIB was associated with recurrent UTI (odds ratio [OR], 3.3; 95% confidence interval [CI], 1.3–9; *P* = .017), especially in children without urinary tract functional or morphological abnormalities (OR, 8.0; 95% CI, 2.3–27.4; *P* = .000). IBCs were associated with lower urinary tract syndrome (OR, 3.6; 95% CI, 1.1–11.8; *P* = .05) and absence of fever (*P* = .009).

Conclusions. IBCs/IIB could explain a high proportion of children with recurrent UTI.

Keywords. urinary tract infection; intracellular bacterial communities; *E. coli* pathogenicity.

Urinary tract infection (UTI) is the most common bacterial infection in children. The risk of suffering a UTI is 3%–7% in girls and 1%–2% in boys until 11 years of age [1–3].

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UTI in children is the main risk factor for the development of renal scarring, arterial hypertension, and renal failure and is a marker of morphological or functional abnormalities of the urinary tract [1, 2].

The development of UTI depends on the characteristics of the microorganism and host-related factors. In fact, the requirements of the presence of virulence genes in uropathogenic *Escherichia coli* (UPEC) are determined by the host genetic background and the anatomic and functional conditions of the urinary tract.

Escherichia coli is the most common etiological UTI agent, being responsible for 80%–90% of the cases [2]. Based on the strain's genetic contents, *E. coli* presents 4 phylogenetic groups named A, B1, B2, and D [4]. In

general, extraintestinal *E. coli* and mainly UPEC strains belong to the B2 group (and less frequently to group D), whereas strains from the intestinal microbiota belong to groups A and B1 [4, 5]. The ability of *E. coli* to produce UTI depends in part of the presence of different virulence factors (adhesins, capsule, siderophores, and toxins, among others) [6]. The presence of ≥2 of the following virulence factors genes would define the strains as UPEC: *papA*, *papC* (encode for P pili), *sfa/focDE* (S and Dra fimbriae), *afa/draBC* (afimbrial adhesin), *iutA* (siderophore aerobactin), and *kpsMT* II (type II capsule) [7].

Two pathogenic mechanisms allow microorganisms to reach the urinary tract: the ascending route, which is the most frequent one, in which microorganisms of the intestinal microbiota colonize the periurethral space and ascend through the urethra to the bladder and eventually the kidneys, and the hematogenous route [8]. In the bladder, *E. coli* adheres to the uroepithelium by means of fimbriae, setting off an inflammatory response [8].

Recently, new insights in the pathogenesis of *E. coli* UTI have emerged. UTI models in mice and more recently in humans describe the presence of intracellular bacterial communities (IBCs) in the bladder epithelium [9–15]. Several in vivo and in vitro models have demonstrated that *E. coli* could invade the superficial urothelial cells in early infection stages and after that rapidly multiply in their interior. This brings phenotypical changes in bacteria that result in the establishment of an IBC, and evolution through different stages ending with the formation of structures similar to biofilms [14–16]. Therefore, *E. coli* could escape from the immune response and resist antibiotic-based treatments with scarce intracellular penetration [16–18]. Intracellular bacteria (IB) could emerge from these reservoirs, usually adopting a filamentous morphology and eventually initiating a new IBC cycle [15, 16, 18, 19]. Additionally, neutrophils seem to lose activity in contact with these filaments [14–16]. Recently, Schwartz et al have described that the increase of IBC formation during the acute stage of UTI was associated with chronic cystitis [20].

Even though these results were found in a mouse model, few reports demonstrate the presence of IBC and filamentous bacteria in urine from women and children with UTI [12, 13].

In relation with the host, the risk factors associated with UTI are sex (female), vesicoureteral reflux (VUR), noncircumcision, sphincter control learning stage, voiding dysfunction, obstructive uropathy, urethral instrumentation, and constipation, among others [1]. Hsieh and collaborators reported a rate of 14% of urological abnormalities among hospitalized children with UTI, being VUR the most common [21]. In Uruguay, 40.7% of children between 0 and 14 years of age who were hospitalized because of UTI presented with VUR [22]. However, most children with UTI do not have any underlying functional or anatomic defects. Innate rather than adaptive immunity is essential for bacterial clearance during UTI. Genetic alterations that reduce Toll-like receptor 4 function are associated with long-term bacterial colonization and asymptomatic bacteriuria, whereas polymorphisms reducing interferon regulatory factor 3 or chemokine (C-X-C motif) receptors 1 expression are associated with acute pyelonephritis and an increased risk for renal scarring [23, 24].

Once the first episode of UTI occurs, between 12% and 20% of children will exhibit recurrences in the following 12 months [25, 26]. VUR is present in 13%–40% of children with recurrent UTI and is considered the most important risk factor [26, 27]. The presence of IBCs could be the base of a new recurrent UTI pathogenic model.

The objective of this study was to determine the presence of IB in children with *E. coli* UTI, to characterize the virulence attributes of *E. coli* strains, and to analyze the relation between IB and the clinical outcome of the patients, especially with recurrent UTI.

MATERIALS AND METHODS

Children with *E. coli* UTI who were seen in the Paediatric Hospital, Centro Hospitalario Pereira Rossell, between June and November of 2012 were included. Urine samples that provided significant *E. coli* growth were processed for the detection of IB. This study was approved by Ethical Committee of Facultad de Medicina Universidad de la República Uruguay (Exp. No. 071140-001118-08).

Detection of Intracellular Bacteria
Samples of 500 μL–1 mL of urine were cytocentrifuged for 6 minutes at 2000 rpm onto glass slides. Samples were then heat-fixed and stained following the Wright–Giemsa method and formalin-fixed for immunofluorescence staining. Samples were analyzed by light microscopy for the detection of dark staining cells with images suggestive of IB or filamentous bacteria as described by Rosen et al [12].

The confirmation of IB was made by confocal laser scanning microscopy searching for the presence of bacteria within desquamated epithelial cells in urine [12]. IBC was defined when ≥5 bacterial clusters were found in the cellular interior. Immunofluorescence staining was performed according to the protocol previously published [13] using specific rabbit anti–*E. coli* antibody coupled to fluorescein isothiocyanate (Abcam), goat anti–uroplakine III (UPIII, Santa Cruz biotechnology) primary antibodies, and Alexa Fluor 568 donkey antigoat immunoglobulin G secondary antibody (Molecular Probes). Slides were fixed with 4% of paraformaldehyde for 15 minutes. After washing with phosphate-buffered saline (PBS), UPIII (1/50) staining was performed during 15 minutes and then cell permeabilization was performed. After 15 minutes, slides were washed and incubated with the *E. coli* antibody (1/50) and Uroplakine III

(1/50) for 1 hour at room temperature. Then, the samples were washed with PBS and incubated with donkey antigoat antibody (1:400) for 30 minutes. Once staining was finished, the slides were mounted with 10 μL of Citifluor. Acquisition and processing of 3-D image stacks were performed as described before using 350/460, 488/520, and 543/565 excitation/emission wavelengths [28]. Acquisition step size was of 0.3 μm in the z axis and 1024 × 1024 pixels in the x-y plane with a pixel size of 70 nm. The 3-D image stack was deconvolved using the Huygens Software and were reconstructed using Volocity 3-D Image Analysis Software (PerkinElmer). All the urine samples were analyzed by confocal microscopy.

Characterization of *E. coli* Strains
Escherichia coli phylogenetic groups A, B1, B2, and D were determined by multiplex polymerase chain reaction (PCR) according to Clermont et al [4].

Virulence factor genes were detected by multiplex PCR. First, 6 virulence factors genes were searched according to Johnson et al [7]: *papA* (P pili, 717 bp), *papC* (P pili, 205 bp), *sfa/focDE* (S and Dra fimbriae, 410 bp), *afa/draBC* (afirmbrial adhesin, 594 bp), *iutA* (siderophore aerobactin, 302 bp), and *kpsMT* II (type II capsule, 272 bp). Those isolates that showed ≥2 of these virulence factors genes were considered UPEC. Five other multiplex PCR tests to detect 19 additional virulence factors were performed according to Johnson and Stell on UPEC strains [29]: PAI (pathogenicity island, 925 bp), *fimH* (type 1 pili, 508 bp), *kpsMT* III (type III capsule, 392 bp), *papEF* (P pili, 326 bp), *ibeA* (invasin,171 bp), *fyuA* (siderophore, 787 bp), *papG* alel III (P pili, 258 bp), K1 (capsule,153 bp), *hlyA* (hemolysin A, 1177 bp), *rfc* (gene involved in synthesis of O4 antigen, 788 bp), *nfaE* (nonfimbrial adhesin, 559 bp), *cvaC* (colicina V, 697 bp), *focG* (F1C fimbriae, 364 bp), *traT* (serum survival, 290 bp), *papG* allele II (P pili 190 bp), *papG* allele II and III (P pili 1070 bp), *cnf1* (toxin, 498 bp), *sfaS* (S fimbriae, 244 bp), and K5 (capsule,159 bp).

Clinical Features of Patients With *E. coli* UTI
Clinical data of patients with and without IB were retrospectively collected by revision of medical records. Variables collected were age, sex, symptoms and signs, urine exam results, whether it was the first episode of UTI or recurrence, and presence of morphological or functional abnormalities of the urinary tract.

Statistical Analysis
The association between clinical features of the patients, recurrence of UTI, phylogenetic group, and virulence factors with the presence of IB was analyzed. Numeric variables were expressed as standard deviation. The χ^2 test or Fisher exact test were applied to nominal variables. P values <.05 were considered significant. Odds ratios (ORs) were calculated with 95% confidence interval (CIs). Statistical analyses were performed using SPSS 17.0 software (IBM SPSS Inc, Chicago, Illinois).

RESULTS
One hundred thirty-three children with UTI were included in the study. Seventy-four clinical records were analyzed, whereas the remaining 59 were not included because of incomplete clinical data.

Clinical Characteristics of Children With UTI
The mean age was 4 years, ranging between 3 months to 14 years; 68 were female (91.9%).

Fever was the most common clinical presentation, seen in 50 cases (67.6%); in 20 cases it was the only symptom observed. Twenty patients showed lower urinary tract symptoms (27%). Other symptoms were abdominal pain (12 cases), vomiting (9 cases), fetid urine (12 cases), and diarrhea (4 cases), all of which were associated with fever and/or lower urinary tract symptoms. Four patients presented poor weight gain as the only sign. Twenty-eight patients (37.8%) showed at least 1 previous UTI episode, and only 7 of them presented with a double excretory tract or VUR.

E. coli Strain Characterization
Escherichia coli distribution between phylogenetic groups was as follows: 102 strains belonged to the B2 and D groups (76.6%) and among these, 59 of them belonged to group D and 43 to group B2. Concerning the rest of the strains, 23 were included in group A (17.2%) and 8 in group B1 (6%).

One hundred six strains (79.6%) were considered to be UPEC according to the detection of ≥2 virulence factors after the screening PCR, and 89 of them belonged to groups B2 or D ($P = .000$). From 27 non-UPEC strains, 11 belonged to phylogenetic group D (40.7%), 8 to A (29.6%), 6 to B1 (22.2%), and 2 to B2 (7.4%).

Table 1 shows the frequency of the most important virulence factors detected in UPEC and non-UPEC strains and their association with the presence of IB. All the virulence factor genes included in the screening PCR were statistically absent in the non-UPEC strains (*afa/draBC*: OR, 1.96 [95% CI, 1.62–2.36], $P = .000$; *sfa/focDE*: OR, 1.37 [95% CI, 1.22–1.54], $P = .001$; *kpsMTII*: OR, 18.3 [95% CI, 6.4–52.1], $P = .000$; *papA*: OR, 39.6 [95% CI, 5.17–303.1], $P = .000$; *papC*: OR, 16.9 [95% CI, 4.76–60.1], $P = .000$; *iutA*: OR, 15.6 [95% CI, 5.6–43.8], $P = .000$).

Association of Intracellular Bacteria With Virulence Factors and Clinical Features of Children With UTI
In 49 of 133 (36.8%) urine samples, the presence of IB was suspected after optical microscopic analysis. In 3 of them, the presence of dark staining cells containing what appeared to be IB were observed, and in the other 46 the presence of filamentous bacteria (with or without cells suggestive of IB) were identified (Figure 1).

Table 1. Frequencies of the Main Virulence Factors Detected in Uropathogenic *Escherichia coli* (UPEC) and Non-UPEC Strains According to Presence of Intracellular Bacterial Communities/Intracellular Bacteria

Virulence Factor Genes	UPEC Strains (n = 106)				Non UPEC Strains (n = 27)			
	IBCs/IIB, No. (%) (n = 34)	No IBCs/IIB, No. (%) (n = 72)	Total, No. (%) (n = 106)	P Value	IBCs/IIB, No. (%) (n = 15)	No IBCs/IIB, No. (%) (n = 12)	Total, No. (%) (n = 27)	P Value
Screening gene virulence factors								
papA	19 (56)	45 (62)	64 (60)	.53	1 (6.6)	0	1 (3.7)	1
papC	22 (64)	50 (69)	72 (68)	.66	1 (6.6)	2 (16.6)	3 (11)	.56
iutA	31 (91)	66 (92)	97 (91)	1	5 (33.3)	6 (50)	11 (41)	.45
kpsMTII	28 (82)	61 (84)	95 (89)	.78	6 (40)	0	6 (22)	.02
afa/draBC	12 (35)	40 (55)	52 (49)	.06	0	0	0	. . .
sfa/focDE	9 (26)	20 (28)	29 (27)	1	0	0	0	. . .
fimH	27 (79)	66 (92)	93 (87)	.11				
papGII	21 (62)	45 (62)	66 (62)	1				
fyuA	30 (88)	68 (94)	98 (92)	.26				
papEF	19 (56)	45 (62)	93 (88)	.51				
papG II-III	19 (56)	43 (60)	50 (47)	.67				
traT	26 (76)	52 (72)	78 (73)	.81				

The χ^2 test was applied to nominal variables. $P < .05$ was considered significant.

Abbreviations: IBCs, intracellular bacterial communities; IIB, isolated intracellular bacteria; UPEC, uropathogenic *Escherichia coli*.

Intracellular bacteria were detected by confocal microscopy in 49 of 133 (36.8%) samples, appearing in 2 different forms: IBCs in 30 cases and as isolated intracellular bacteria (IIB) in the other 19 (Figure 2 and Supplementary Video). Considering the 49 samples with IBCs/IIB detected by confocal microscopy, only 21 of them exhibited filamentous bacteria (42%).

Comparing the results between light and confocal microscopy, coincidence was observed only in 85 samples, 25 with IBCs/IIB and 60 without IBCs/IIB. Twenty-four cases of IBCs/IIB were not detected by light microscopy.

Fifteen of the 49 strains with IBCs/IIB catalogued as non-UPEC (30.6%) were found at an intracellular level ($P = .028$).

Within the UPEC group, any of the assessed virulence factors was statistically associated with the presence of IBCs and/or IIB, whereas in the non-UPEC group, kpsMTII was associated with the presence of IBCs/IIB (Table 1).

Clinical data of the 74 patients were analyzed, including 34 with IBCs/IIB (cases) and 40 without IBCs/IIB (controls). Clinical and laboratory features of patients were shown to be related to the presence or absence of IBCs/IIB and to the presence or absence of filamentous bacteria (Table 2).

The presence of IIB and/or IBCs was statistically associated with recurrent UTI (OR, 3.3 [95% CI, 1.3–9], $P = .017$), with higher risk if the patient did not present morphological or
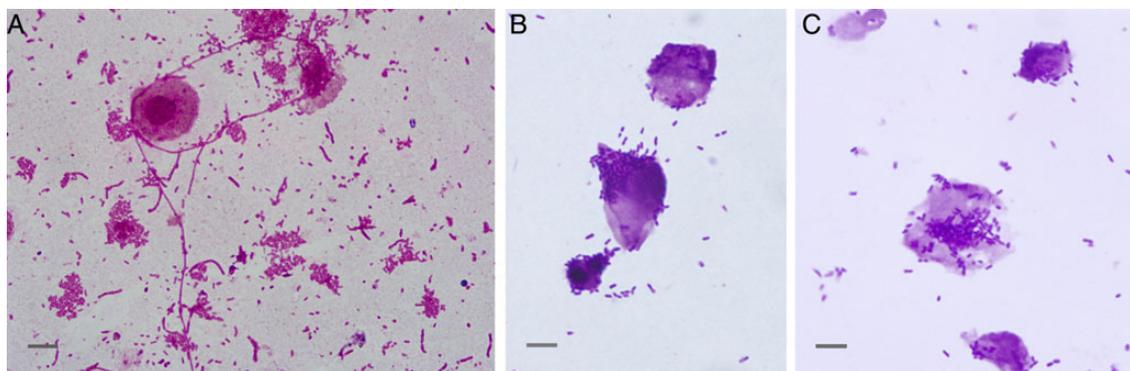

Figure 1. Filamentous bacteria and exfoliated urothelial cells in urine samples suspicious for intracellular bacterial communities/isolated intracellular bacteria by light microscopy. Light microscopic images of exfoliated uroepithelial cells stained with Wright–Giemsa. *A*, Detached urothelial cells and filamentous bacteria. *B* and *C*, Dark stained cells with suspicious IBCs. Scale bar = 5 μm. Abbreviation: IBCs, intracellular bacterial communities.
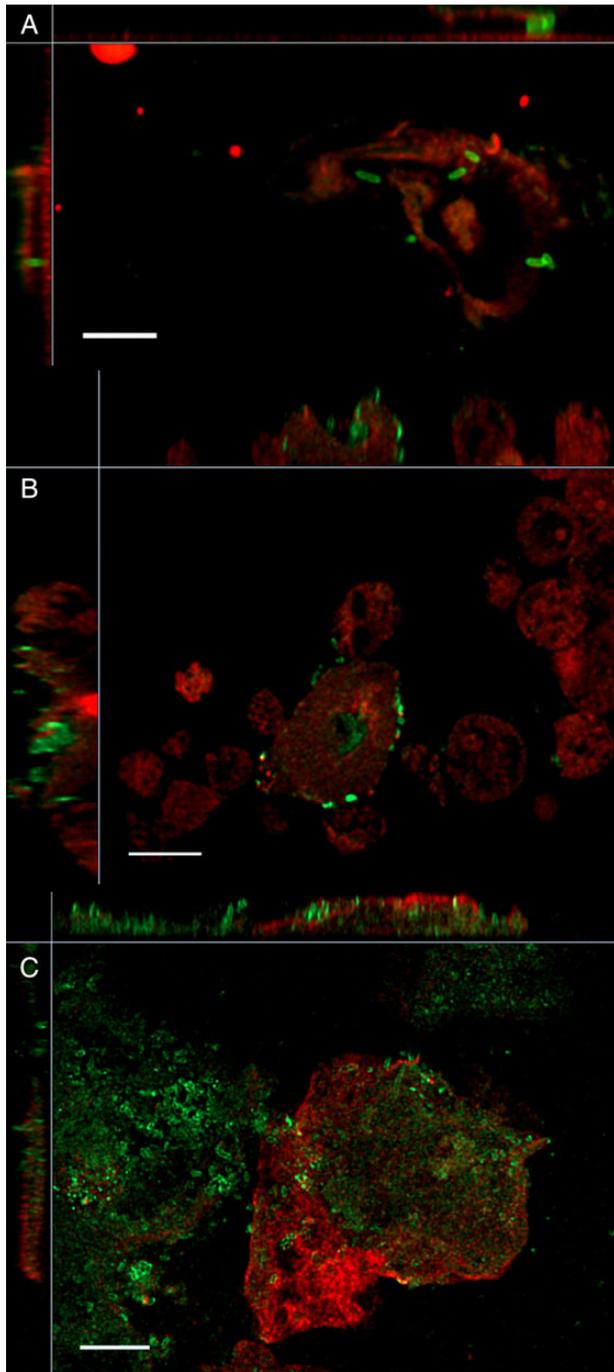
Figure 2. Detection of intracellular bacterial communities (IBCs)/intracellular bacteria (IB) by confocal laser scanning microscopy in exfoliated urothelial cells in urine from children with *Escherichia coli* urinary tract infection. Confocal laser scanning microscopic images of exfoliated uroepithelial cells stained with uroplakine III primary antibodies (structural protein of bladder epithelial cells in red), Alexa Fluor 568 donkey antigoat immunoglobulin G secondary antibody, and specific antibodies for *E. coli* (green). Scale bar = 7 μm. *A*, Isolated IB. *B*, Small IBCs. *C*, IBCs.

functional disorders of the urinary tract (OR, 8 [95% CI, 2.3–27.4], *P* = .000). IBC formation was statistically associated with the presence of lower urinary tract symptoms (OR, 3.6 [95% CI, 1.1–11.8], *P* = .05) and absence of fever (*P* = .009). IIB did not show any correlation with clinical symptoms. No association with clinical presentation or recurrence was observed when filamentous bacteria were detected (Table 2).

DISCUSSION

The ability of *E. coli* to invade bladder epithelial cells has been recently reported, and a few studies have described the presence of IB in detached urothelial cells in human urine [12, 13]. Intracellular reservoirs allow these microorganisms to avoid the action of the immune response and antibiotics such as β-lactams [14–17, 30]. These *E. coli* could persist in the urinary tract and be the cause of recurrent UTI.

In the population of children included in this study, 28 (37.8%) had had previous UTI, with only 8 cases (28.5%) related to the presence of urinary tract abnormalities. Of the remaining 20, 16 (57.1%) could be explained by the presence of IB (OR, 8 [95% CI, 2.3–27.4]).

In 46 of the analyzed cases, it was the first episode of UTI; IB were found in 16 of them. Given that the clinical records were analyzed retrospectively, we could not establish if any of these patients developed new UTI. Prospective case-control studies would be necessary to establish the role of IB in recurrent UTIs.

In murine models, different maturation stages could be observed along the IBC generation cycle. During the first 1–3 hours after infection, a single bacterium invades the urothelium and replicates, forming early IBCs of rod-shaped cells in loosely organized colonies. The next IBC stage occurs 6–8 hours after infection, when IBCs adopt a tightly packed cluster of coccoid bacteria. Approximately 16 hours after infection, in the late IBC stage, bacteria differentiate into rod-shaped cells and start detaching from the IBCs. These bacteria could escape from the superficial epithelial cell and start a new IBC formation cycle, or be expelled from the host by micturition [15].

In this study, 2 different forms of IIB were described: IIB or IBCs. Both forms were associated with recurrent UTI. These forms could be different stages of the same process, IIB probably corresponding to the initial stages of IBC formation (Figure 2*A*). As observed in Figure 2*B* and 2*C*, bacteria have a small size and a coccoid shape consistent with the observations of the midstage IBCs in the mouse model [15].

After the late stages in IBC formation, filamentous bacteria are observed on the luminal surface of the bladder [15, 18]. Filamentous UPEC could be associated with higher attachment ability to the urothelial cells through the increase in adhesion contact points, explaining the apparent difficulty faced by phagocytes in clearing filaments and bacterial resistance against strong urine shear forces [18, 19, 31].

Table 2. Clinical Features of Patients Related to the Presence or Absence of Intracellular Bacterial Communities/Intracellular Bacteria and Filamentous Bacteria

Characteristic	No IBCs/IB (n = 40)	IBCs/IB (n = 4)	P Value	IBCs (n = 20)	P Value	IB (n = 14)	P Value	Nonfilamentous (n = 45)	Filamentous (n = 29)	P Value
Female sex	36	32	.68	19	.65	13	1.00	40	28	.39
Fever (≥38°C)	31	19	.08	8	.009	11	1.00	29	21	.61
Lower UTI symptoms	8	12	.19	9	.05	3	1.00	14	6	.42
Recurrent UTI	10	18	.02	10	.08	8	.04	20	8	.14
Recurrent UTI without urinary tract anomalies	4	16	.00	9	.00	7	.04	14	6	.29
Urinary tract anomalies	7	2	.28	1	.42	1	.65	7	2	.46
Nitrites	18	9	.09	5	.16	4	.35	18	9	.46
Leukocytes	29	23	.60	12	.37	11	1.00	32	19	.44

Abbreviations: IB, intracellular bacteria; IBCs, intracellular bacterial communities; UTI, urinary tract infection.

Few studies have sought the presence of IBCs in exfoliated urothelial cells in humans [12, 13]. Rosen et al found a significant correlation between light and confocal microscopy for the detection of IBCs; however, they performed confocal microscopy only in urine samples positive by light microscopy [12]. In this study, we sought for the presence of IBCs by confocal microscopy in all urine samples, allowing the detection of almost 50% of IBCs/IIB that were not detected by light microscopy, mainly because of the presence of IIB.

Although Rosen et al described the presence of filamentous bacteria in 100% of the samples with IBCs [12], in our study filamentation was observed just in 42% of the urine samples with IBCs/IIB. This may be because filamentation is a transient process and septation must be restored to initiate the following round of IBC formation during acute infection [18, 19]. In this respect, Andersen et al observed that filament reversal started within 30 minutes to 1 hour after harvest and continued during the following 3–4 hours [19]. In our study, urine samples were processed for microscopy analysis between 12 and 24 hours after micturition; thus, filamentous forms could have reverted to rod-shaped bacteria.

It is interesting to note that 15 of the 49 strains that were detected intracellularly were not classified as UPEC according to classical definitions based on the presence of certain virulence factors and phylogenetic groups. Considering only the presence of virulence factors, these isolates could be considered as colonizers. However, these bacteria were intracellular, detected within the bladder cells, and related to recurrent UTI. Thus, defining UPEC just according to the presence of certain virulence factors would underestimate a group of E. coli that could cause UTI as well. This probably could reflect the diversity of UPEC.

Most of the UTI therapeutic guidelines for children recommend the use of β-lactams for empirical treatments. However, these antibiotics reach low intracellular concentrations, so it would be necessary to provide an alternative therapeutic plan in patients with recurrent UTI without anatomical or functional urinary tract pathology in which IIB or IBCs may be involved [32, 33]. Blango et al found that only a few antibiotics, including nitrofurantoin, ciprofloxacin, and sparfloxacin, were able to eliminate IB in bladder cell culture–based assays. However, in a mouse UTI model, UPEC reservoirs in bladder tissues were not effectively eradicated when using these antibiotics [17]. New studies in mouse models propose the use of different agents to expel bacteria from the cell-like chitosan (a bladder cell exfoliant) that in association with antibiotics would eradicate intracellular reservoirs or protamine sulfate, a highly cationic protein that removes bound and intracellular UPEC, causing exfoliation of umbrella cells [34, 35]. Treatment with forskolin, a drug that increases intracellular cyclic adenosine monophosphate levels, expels UPEC from the intracellular reservoir, rendering the bacteria susceptible to immune responses and antibiotics [24].

Prospective studies involving clinical and basic research will be necessary to elucidate the importance of different therapeutic strategies to eradicate IB in recurrent UTI in humans.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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