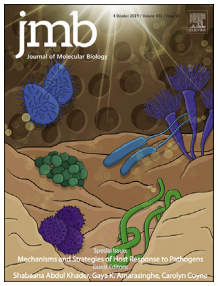

Direct Detection of Tissue-Resident Bacteria and Chronic Inflammation in the Bladder Wall of Postmenopausal Women with Recurrent Urinary Tract Infection

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Abstract

Urinary tract infections (UTIs) are the most commonly reported infections in adult women and have high rates of recurrence, especially in postmenopausal women. Recurrent UTI (RUTI) greatly reduces quality of life, places a significant burden on the healthcare system, and contributes to antimicrobial resistance. Because treatment of RUTI by long-term antibiotic therapy is often ineffective or poorly tolerated in elderly women, new therapies must be developed. The molecular basis of RUTI, especially in postmenopausal women, has remained unclear because modeling RUTI in mice is difficult, and human data are limited. Invasion of the urothelium and induction of host inflammation are hypothesized to be key mechanisms by which bacterial pathogens cause RUTI. To further our understanding of RUTI in humans, we performed a systematic analysis of urine and bladder biopsy samples from postmenopausal women undergoing cystoscopy with fulguration of trigonitis in the advanced management of antibiotic-refractory RUTI. We provide direct evidence that bacteria reside in the bladder wall of postmenopausal RUTI patients and that diverse bacterial species can be isolated from the bladder tissue. Histopathological scoring revealed significant edema and alterations of urothelial architecture in RUTI patient biopsies. Lymphocytes, including plasma B-cells, were detected within the mesenchyme, urothelium, and follicular aggregates in the majority of patients, indicating that the local adaptive immune response is active during human RUTI. These data provide conclusive evidence that bacteria invade the human urothelium and suggest that diverse bacterial species and the adaptive immune response play important roles in RUTI in humans.

Introduction

Each year, at least 150 million women worldwide will suffer from a urinary tract infection (UTI) [1]. Because of their prevalence, the societal impact of UTIs is high and their treatment costs billions of dollars annually [2]. UTIs are among the most common primary diagnoses for women visiting emergency rooms in the United States [3]. UTIs are categorized as uncomplicated or complicated and either lower urinary tract (cystitis) or upper urinary tract (pyelonephritis) [4]. *Uncomplicated UTIs* occur in otherwise healthy individuals who have no urinary tract abnormalities or obstructions that would predispose them to infection [5]. UTIs are complicated when the patient has urinary tract abnormalities, a compromised immune system, or an indwelling urinary catheter [6]. Antibiotic therapy

is the primary and oftentimes only treatment prescribed for uncomplicated UTI in women of all ages. Consequently, UTI is the most common indication for prescription of antibiotics to older adults [7]. Even with antibiotic treatment, UTIs have a high rate of recurrence, ranging from 19% to 36% in premenopausal women and increasing to 55% in postmenopausal women [8–11]. Recurrent UTI (RUTI) is defined as $\geq$3 uncomplicated UTIs within 12 months or 2 uncomplicated, symptomatic UTIs within 6 months [12]. One of the primary risk factors of RUTI, besides previous infection, is advanced age, with 10%–15% of women over the age of 60 years experiencing RUTI [13]. Although RUTI disproportionally affects postmenopausal women, the disease has been understudied in this population, and little is known about its etiology [13]. Proposed contributing factors include altered estrogen status as well as the chronic nature of the infection and associated inflammation [14–16].

UTIs are caused by diverse gram-negative and/or gram-positive bacteria and less commonly fungi. The causative agent of most UTIs, including RUTI, is uropathogenic *Escherichia coli* (UPEC). However, the incidence of non-*E. coli* UTI is higher in RUTI patients than in acute UTI patients [17]. Following UPEC, common UTI pathogens include *Klebsiella pneumoniae*, *Staphylococcus* spp., *Enterococcus faecalis*, group B *Streptococcus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Candida* spp. [3]. Studies in mice have revealed that a key pathogenic mechanism of UPEC is the ability to form intracellular bacterial communities (IBCs) within the superficial cells of the bladder epithelium, also termed umbrella cells [18,19]. Within IBCs, bacteria are recalcitrant to antibiotic treatment and can quickly multiply and disperse to invade other cells. In mice, UPEC also can establish quiescent intracellular reservoirs (QIRs) consisting of 4–10 non-replicating bacteria in the transitional layer of the bladder epithelium [20–22]. Two, not mutually exclusive, hypotheses describe how RUTIs occur, the first being reinfection from a second translocation event of gut-resident uropathogens into the urinary tract. The second hypothesis is that RUTIs are caused by QIRs that become re-activated and serve as the seed for a new infection [20]. However, to date, IBCs or QIRs have not been directly detected within the bladder wall of human RUTI patients. In addition, studies in mice have implicated the immune response as a predisposing factor for recurrent infection [23–27]. Although much is known about the immunological landscape of the bladder during RUTI in mice, little is known about the nature of the local innate and adaptive immune response in human RUTI patients [26–28].

In this work, we performed a systematic analysis of urine and bladder biopsies from 14 postmenopausal women undergoing cystoscopy with fulguration of trigonitis (CFT) for the treatment of antibiotic-refractory RUTI. We cultured diverse bacterial species from both the urine and bladder tissue of these patients. 16S rRNA fluorescence *in situ* hybridization (FISH) of the RUTI patient biopsies revealed that bacteria do form communities resembling both IBCs and QIRs within the host bladder epithelium, and in some patients, within even deeper layers of the bladder wall. Histopathological analysis of the patient biopsies indicated that, in addition to the expected acute inflammatory response, there was also evidence of an active adaptive cellular and humoral immune response within the bladder epithelium of human RUTI patients. This work provides an important benchmark for future work aiming to understand both the bacterial pathogens and the host immune factors that contribute to RUTI in postmenopausal women.

Results

Study design and patient characteristics

As part of institutional review board-approved studies, ‘‘Effectiveness of fulguration in the treatment of recurrent urinary tract infections due to trigonitis’’ STU 082010-016, ‘‘Bacterial study form urine of postmenopausal patients with recurrent UTI’’ STU 032016-006, and ‘‘Analysis of Bacteria Associated with Recurrent UTIs’’ MR 17-120, patients who had elected CFT for the advanced management of antibiotic-refractory RUTI were recruited for urine and biopsy collection. CFT is a treatment for RUTI in which areas of chronic cystitis in the bladder, presumably harboring intracellular bacteria, are fulgurated with an electrode during cystoscopy while the patient is under anesthesia. CFT is thought to physically remove the inflamed tissue as well as intracellular bacteria or bacterial biofilms that are otherwise protected from antibiotic therapy. In one study, the efficacy rate of CFT in eliminating antibiotic refractory RUTI was reported to be 65%–75% over time [29]. Urine and biopsies were obtained from 14 postmenopausal female patients who met strict inclusion criteria for uncomplicated RUTI. Patient characteristics and urine culture results prior to CFT are described in Table 1. Briefly, all patients were postmenopausal, ranged in age from 53 to 86 years (median, 70.5 years) and had body mass indexes ranging from 20 to 35.7 (median, 29.9 years). Out of the 14 patients, 5 had previously undergone at least one CFT procedure, and all had positive clinical urine cultures immediately preceding the CFT, except PNK010 and PNK014 who were on long-term prophylactic antibiotic treatment (Table 1).

Table 1. Patient characteristics

Patient	Age (y)	Diabetes	BMI	Prior CFT	Urine culture history	Urine culture (CFU/mL) before CFT
PNK001	67	No	32.9	Yes (1)	*E. coli*, *Proteus*, *E. faecalis*	*P. aeruginosa* ($5–9.9 \times 10^4$)
PNK002	72	No	35.7	No	*E. coli*, *M. morganii*	*E. coli* (10^4), *M. morganii* (10^4)
PNK003	69	No	30	Yes (1)	*K. pneumoniae*	*K. pneumoniae* (10^5), *P. aeruginosa* ($1–4.9 \times 10^4$), *E. faecalis* ($1–4.9 \times 10^4$)
PNK004	68	No	24.5	Yes (4)	*E. coli*, *E. faecalis*	*E. coli* (10^5)
PNK005	69	No	25.9	No	*E. faecalis*, *P. rettgeri*, *P. aeruginosa*	*M. morganii* (10^4)
PNK006	83	AODM	36.1	No	*E. coli*	*E. coli* (10^5)
PNK007	86	IDDM	25.1	No	*E. coli*	*E. coli* ($1–4.9 \times 10^4$)
PNK008	73	No	31.8	No	*K. pneumoniae*, *E. coli*, *E. faecalis*	*K. pneumoniae* ($1–4.9 \times 10^4$)
PNK009	78	No	22	Yes (1)	*E. coli*	*E. coli* (10^5)
PNK010	54	No	25.9	No	NR	Culture-negative
PNK011	61	No	31.6	No	*E. faecalis*, *E. coli*	*E. faecalis* ($1–2.5 \times 10^4$)
PNK012	81	No	20	No	*S. epidermidis*	*S. epidermidis* ($5–9.9 \times 10^4$)
PNK013	76	No	29.9	No	*E. coli*	*E. coli* (10^5)
PNK014	53	No	36	Yes (1)	*K. pneumoniae*	Culture-negative

Relevant patient data recorded for the 14 study participants. Diabetes: No, non-diabetic; NR, no record; CFT, cystoscopy with fulguration of trigonitis with number in parentheses indicating number of prior CFT procedures; IDDM, diabetes melitus type 1; AODM, adult-onset diabetes melitus type 2. Results of urine culture most immediately preceding the CFT treatment (day 0 in Fig. 1a). Preceding urine cultures performed in clinical laboratories with a 10^4 CFU/mL detection limit.

The timeline for patient recruitment, antibiotic treatment, sample collection, and CFT procedure is illustrated in Fig. 1a. On day 0, cystitis was confirmed via in-office cystoscopy. Following election of the CFT procedure, patients were recruited to the study. The CFT procedure was scheduled 4–6 weeks from the initial office visit. Patients were prescribed antibiotics prophylactically while waiting for the CFT and were asked to stop antibiotic therapy 1 week prior to the CFT procedure. On the day of the CFT procedure, patients were first anesthetized and then direct bladder urine was collected and immediately placed on ice. Next, up to six cold cup bladder biopsies were taken, two from regions that were visibly normal [control 1 (C1) and control 2 (C2)] and four from regions of visible cystitis [infected 1–4 (I1–I4)]. Biopsies were placed directly in in 4% paraformaldehyde or in an antibiotic solution of penicillin (Pn) and gentamicin (Gm; Fig. 1b).

To culture intracellular, intra-tissue or biofilm-associated bacteria from patient biopsies C2 and I1–I4, the biopsies were incubated in the 100 μg/mL Pn/Gm solution for 2 h to kill extracellular bacteria (Fig. 1b). The combination of Pn/Gm was used because Pn has been found to act synergistically with Gm to effectively kill resistant *E. faecalis* [30]. To ensure extracellular bacteria had been eliminated, the supernatant was plated on BHI agar, and then the tissue was washed thrice in sterile 1× PBS. Biopsies were then homogenized, and host cells were lysed in 0.5% Triton X-100. Lysates and urine were plated both on BHI and Chromagar. Well-isolated colonies were cultured and identified by 16S rRNA gene sequencing.

Diverse bacterial species cultured from patient urine and biopsies

The biopsy and urine culture results are summarized in Table 2, and the complete list of bacterial isolates derived in this study is listed in Table S1. Our culture methods only detected bacteria in the urine of six patients (PNK003–PNK008). The bacteria detected in urine of five of these patients included well-established uropathogens including *K. pneumoniae, E. coli,* and *E. faecalis. E. coli* was present in high concentrations in the urine of three patients ($10^6–2 \times 10^8$ CFU/mL), while *E. faecalis* was detected in the urine of two patients ($10^3–10^5$ CFU/mL), and *K. pneumoniae* (10^4 CFU/mL) was only detected in the urine of a single patient. The biopsy culture results are also presented in Table 2. As expected, all supernatant cultures from the biopsies were negative except for PNK006, indicating that all extracellular bacteria had been eliminated by the Pn/Gm treatment. A possible explanation for the positive supernatant culture for PNK006 was that due to the unusually delicate and buoyant nature of the patient's biopsy tissue, it was not effectively pelleted at 500*g*. Furthermore, the *E. coli* strain isolated from the supernatants of the PNK006 biopsies was sensitive to the 100 μg/mL Pn/Gm treatment, suggesting that extracellular bacteria would not have survived the treatment (data not shown).

The biopsy plating protocol was designed so to isolate bacteria that were either intracellular, intra-tissue, or within a protective biofilm that is very closely associated with the urothelium. The majority of biopsies from visually uninflamed "control" regions

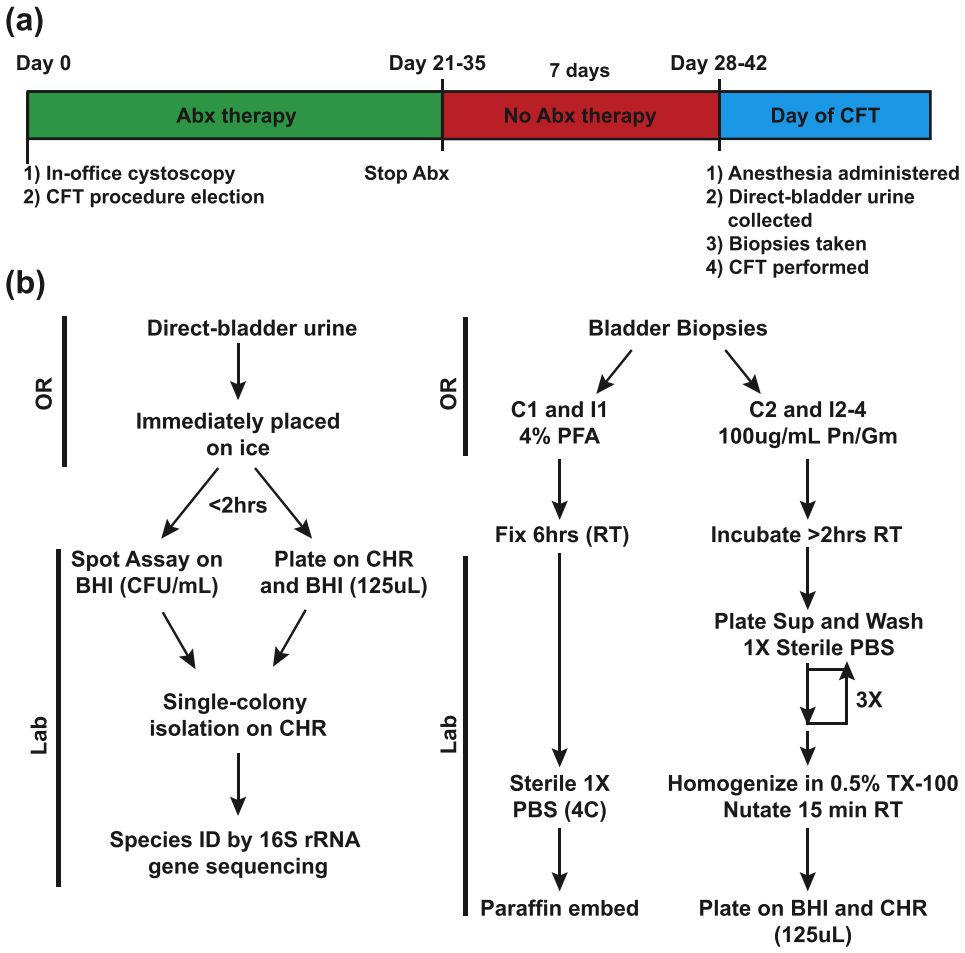

Fig. 1. Patient recruitment timeline and sample analysis workflow. (a) Timeline of RUTI patient recruitment and sample collection during the CFT procedure. (b) Sample processing strategy used for direct-bladder urine (left panel) and bladder biopsies (right panel) from RUTI patients receiving CFT procedure. CFT, cystoscopy with fulguration of trigonitis; Abx, antibiotics; OR, operating room; C1–2, biopsies taken from visibly uninflamed regions; I1–4, biopsies taken from visibly inflamed regions; PFA, paraformaldehyde; Pn/Gm, penicillin and gentamicin.

(C2) were culture negative, with the exception of PNK001, PNK004, PNK006, and PNK007. However, in three of these patients, the cystitis spread beyond the trigone and visibly uninflamed regions were difficult to identify. In these cases, the C2 biopsies were taken from the least visually inflamed areas within the bladder. Diverse bacterial species were cultured from infected region biopsies (I2–4) of 9 patients. These species included canonical uropathogens such as *E. coli* and *K. pneumoniae,* but also bacterial species not typically associated with UTI including three species of *Bacillus* (Table 2). The fact that these species were only isolated from infected region biopsies and never isolated from the supernatant, washes or from control biopsies, suggests that isolation of these species was not a result of contamination during biopsy processing.

Uropathogens often exhibit high rates of antimicrobial resistance, especially in older populations [31]. To explore the antimicrobial resistance profiles of our isolated uropathogens, we performed minimum inhibitory concentration testing for front-line antibiotics trimethoprim–sulfamethoxazole, ciprofloxacin, and nitrofurantoin on select tissue and urine isolates. All *E. coli* and *K. pneumoniae* strains were resistant to at least one antibiotic (Table S2), and the *K. pneumoniae* strains were extended spectrum beta-lactamase producers. Interestingly, the PNK005 *E. faecalis* urine isolate was not resistant to any of the antibiotics tested, while the PNK007 *E. faecalis* urine and tissue isolates were resistant to both trimethoprim–sulfamethoxazole and ciprofloxacin (Table S2).

FISH reveals bacterial communities within the bladder wall

Our biopsy culture data suggest that intra-tissue bacteria are present in the bladder wall of human RUTI patients, but it is formally possible that the

Table 2. Culture results for PNK001–PNK014 urine and bladder biopsies

Patient no.	Urine		Biopsy lysates				
	PRE	Species (CFU/mL)	Sup	C2	Species	I2–4	Species
PNK001[a]	–	NA	–	+	*S. epidermidis*	+	*S. pastueri*
PNK002	–	NA	–	–	NA	+	*S. epidermidis, Paenibacillus* spp., *Curtobacterium* spp.
PNK003	+	*K. pneumoniae* (10^4), *S. warneri* (10)	–	–	NA		*K. pneumonaie*
PNK004[a]	+	*E. coli* (2×10^8)	–	+	*E. coli*	+	*E. coli*
PNK005	+	*E. faecalis* (10^3)	–	–	NA	–	NA
PNK006[a]	+	*E. coli* (2×10^8)	+	+	*E. coli*	+	*E. coli*
PNK007	+	*E. coli* (10^6), *E. faecalis* (10^5)	–	+	*E. faecalis*	+	*S. hominus*, *Bacillus firmus*
PNK008	+	*Methylobacterium adhaesivum* (56)	–	–	NA	–	NA
PNK009	–	NA	–	–	NA	+	*Brevibacillus laterosporus*
PNK010[a]	–	NA	–	–	NA	+	*Staphylococcus hominus*
PNK011	–	NA	–	–	NA	–	NA
PNK012	–	NA	–	–	NA	–	NA
PNK013	–	NA	–	–	NA	+	*Staphylococcus spp.*
PNK014	–	NA	–	–	NA	–	NA

Urine and bladder biopsies from enrolled patients PNK001–PNK014 were cultured and species of isolates identified. Sup, supernatant; C2, biopsy taken from an area with no visual cystitis, typically the dome; I2–4, biopsy taken from an area with visual cystitis, typically the trigone; NA, not applicable. (+) indicates a positive culture result, and (–) indicates a negative culture result.

[a] Patients presented with pancystitis so "control" biopsies taken from least visually infected region.

bacteria we isolated from patient biopsies were within protective biofilms that were closely associated with the luminal surface of the bladder epithelium and not within the bladder wall itself. We therefore performed FISH on patient biopsy sections to determine if intra-tissue bacteria were present in the bladder wall of human RUTI patients. Using universal probes for the 16S rRNA gene [32], we detected bacteria within the bladder wall of 11 patients (Table S3, Figs. 2 and S1). Representative micrographs of biopsies from both infected and control regions of three patients are depicted in Fig. 2. For some patients (e.g., PNK001), bacteria (white arrows) were only detected in the bladder wall from in the biopsies taken from the infected region (Fig. 2a.). However, in the majority of FISH-positive patients, intra-tissue bacteria were detected in both infected and control region biopsies (Table S3, Fig. S1.) even in cases where visibly normal regions were available for biopsy. For example, bacteria were detected within the bladder wall of both the C1 and I1 biopsies from PNK008 and PNK011 (Fig. 2b, c.), but no signal is observed with the scrambled control probe. It is important to note that in the cases of both PNK008 and PNK011, although bacteria were clearly detected by FISH, no bacterial species were cultured from patient biopsies (Table 2). Since the universal 16S rRNA probe has high fidelity to bacterial 16S rRNA and the possibility of nonspecific binding was controlled for with the scramble FISH probe, two hypotheses could explain this observation [32]. First, that all four biopsies used for culture were taken from regions that did not contain intra-tissue bacteria. The second hypothesis is that these intra-tissue bacteria are anaerobic or otherwise not culturable via our methods. Recent findings that the urinary microbiota comprises many bacterial species that cannot be cultured by conventional aerobic culture methods lend support to this hypothesis [33].

Another interesting observation is that although most patients had at least one FISH-positive biopsy (C1 or I1), putative intra-tissue bacteria were cultured from the biopsies of the only three completely FISH-negative patients, PNK003, PNK005, and PNK007 (Tables 2 and S3). It is possible that the I1 biopsies were not taken from regions containing bacteria in these patients. Another possibility is that these patients were in the acute phase of UTI at the time of biopsy and the bacteria cultured were within exfoliated umbrella cells that were pelleted with the biopsy and not in the bladder wall itself. PNK003, PNK005, and PNK007 all had high titers of bacteria in their pre-procedure urine sample, indicating an active, acute-phase UTI (Table 2.). Furthermore, exfoliated epithelial cells containing *K. pneumoniae* were observed in the urine of PNK003 (Fig. S2). Taken together, our data clearly demonstrate that bacteria are present within the bladder wall of human RUTI patients.

Community size and localization of bladder wall-resident bacteria

To ascertain the size and depth of these communities of intra-tissue bacteria, we performed semi-quantitative analysis of bacterial community size and tissue depth by 16S rRNA FISH (Table S4). Our analysis revealed bacterial communities located in many layers of the bladder wall (Fig. 3a, b). Bacterial communities were observed at all layers of the urothelium; from superficial, transitional, and basal epithelial layers. For example, we observed bacterial communities extending through all layers of the

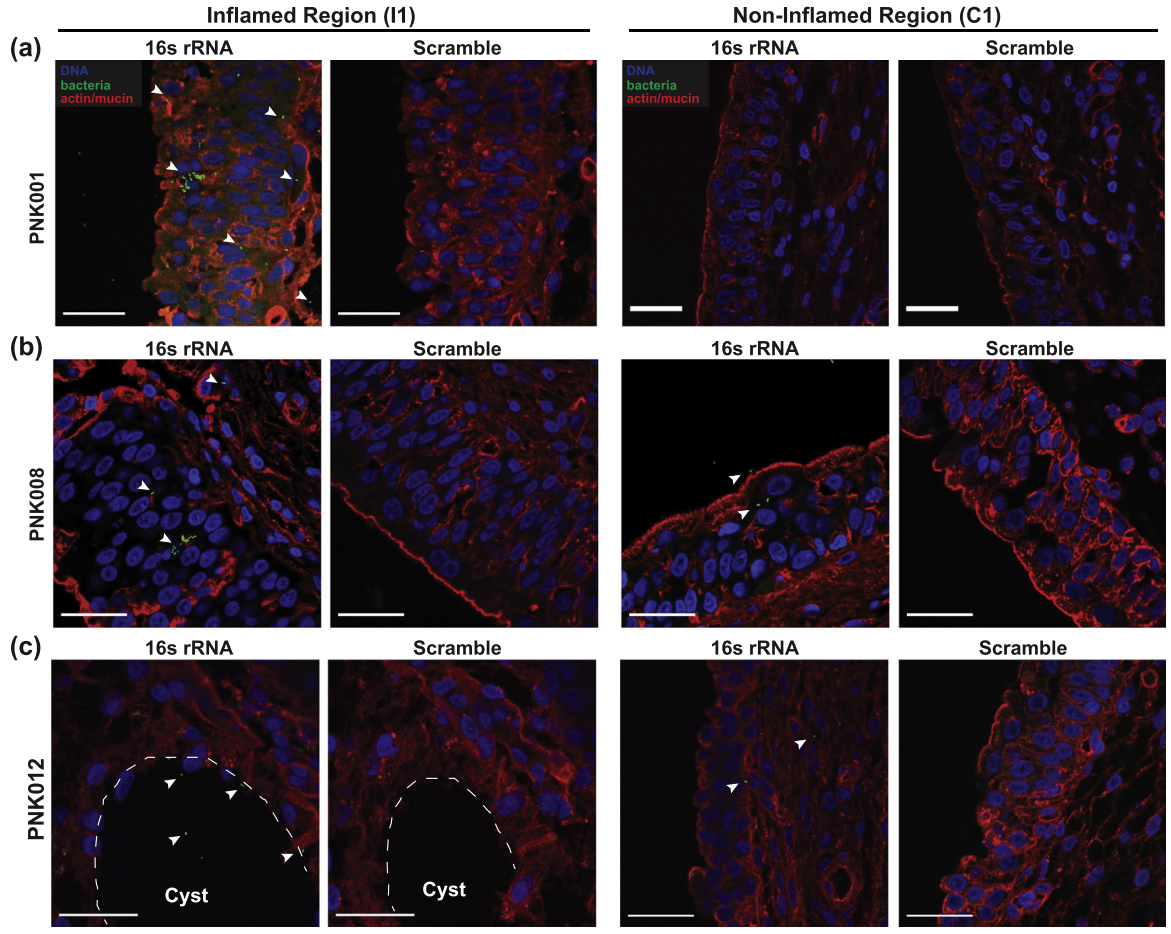

Fig. 2. Detection of intracellular/intratissue bacteria in the RUTI patient bladder wall. Representative FISH micrographs from bladder wall biopsies from visibly inflamed (I1) and visibly uninflamed (C1) regions of three patients, (a) PNK001, (b) PNK008, and (c) PNK012. Inflamed and non-inflamed regions were hybridized with dopo-Alexa488-labeled 16S rRNA universal probe to label bacteria (green) or dopo-Alexa488-labeled scramble probe as a control for non-specific binding. Actin and mucin are denoted in red and cellular nuclei in blue. The scale bar represents 25 μm.

urothelium and within the sub-urolthelial tissue of PNK011 C1 (Fig. 3a). Interestingly, we observed bacteria at varying depths and even within regions that were not visually inflamed. We also observed a proportional enrichment of bacteria in sub-urothelial, mesenchymal regions of the bladder wall in many patient bladder biopsies (Fig. 3b). We attribute these findings to the chronic nature of disease in the patient cohort. The formation of deeper bacterial communities as a function of increasing chronicity of infection has been observed in animal models of UTI [13,22]. Furthermore, our analysis revealed diverse community sizes among the tissue-resident bacteria. The intra-tissue bacteria detected ranged from a single bacterium to larger communities of up to 25 bacteria (Fig. 3c). In most patient biopsies, smaller communities of less than 5 bacteria were more common than larger communities and they were most often found sub-urothelial, mesenchymal regions (Fig. 3c, Table S4).

Histopathological analysis of acute and chronic inflammation in patient biopsies

To begin to understand the immunological landscape of the bladder in postmenopausal women with RUTI, longitudinal sections of C1 and I1 biopsies from all patients were stained with hematoxylin and eosin and then analyzed by a clinical pathologist. A scoring scheme was developed to describe the range of both acute and chronic inflammation observed in the bladder biopsy sections (Table 3). Severity of acute inflammation was defined as the degree of neutrophil infiltration, which has been previously used in similar scoring schemes [34], with a denuded urothelium defining severe acute inflammation. The degree of chronic inflammation was defined by the concentration of lymphocytes within the urothelium and sub-urothelial, mesenchymal regions and is therefore representative of the adaptive immune response. The most severe

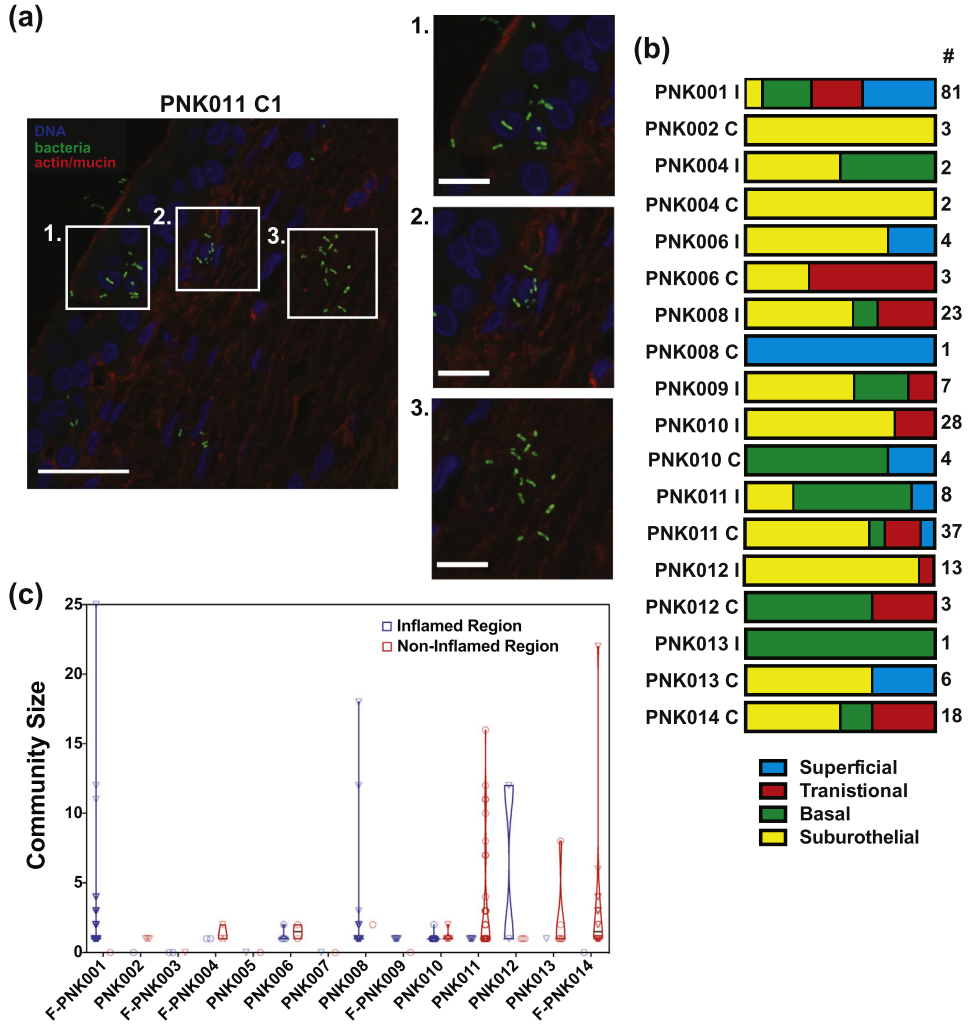

Fig. 3. Diverse community size and tissue localization of bladder-associated bacteria. (a) Representative FISH image of bacterial community depth in RUTI patient bladder wall tissue. 1. Superficial/Transitional epithelium, 2. basal epithelium, 3. suburothelial mesenchymal region. Bacterial 16S rRNA is denoted in green, actin is denoted in red, and cellular nuclei is denoted in blue. The scale bar represents 25 and 10 μm for zoomed detail. (b) Proportional distribution of bacterial community depth in RUTI patient bladder wall tissue. The total number of detected bacterial communities is indicated to the right. Epi, epithelial. (c) Semi-quantitative analysis of bacterial community size in RUTI patient bladder wall tissue. Biopsy tissue collected from trigone is denoted by a triangle, while tissue collected from the bladder dome is denoted by a circle. Patients with a previous history of CFT are denoted with "F."

chronic inflammation was defined by the presence of prominent lymphoid aggregates forming germinal centers.

Pathological analysis of patient biopsies using the devised scoring system revealed that chronic inflammation was evident in all patient biopsy samples.

Table 3. Histopathlogical scoring system for RUTI-associated bladder inflammation

Acute inflammation			Chronic inflammation		
Score	Grade	Histopathologic finding	Score	Grade	Histopathologic finding
0	Normal	Normal	0	Normal	Normal
1	Mild	Neutrophils in mesenchyme	1	Mild	Lymphocytes present
2	Moderate	Neutrophils in urothelium	2	Moderate	Lymphoid aggregates
3	Severe	Denuded urothelium	3	Severe	Prominent lymphoid aggregates

Scoring system for acute and chronic inflammation in RUTI biopsy samples devised through pathological analysis of hematoxylin and eosin-stained patient biopsy sections from inflamed and non-inflamed regions of the bladder.

Lymphoid aggregates were present in either a C1 or I1 biopsy from the majority (11/14) of patients. Acute inflammation defined by neutrophil infiltration was more variable with both C1 and I1 biopsies from three patients (PNK001, PNK009, PNK012) not exhibiting any signs of neutrophil infiltration (Table S5). In addition to scoring the degree of acute and chronic inflammation the presence/absence of edema, changes in urothelial structure, and specific immune cell types were recorded for all bladder biopsy sections (Table S5). Representative micrographs of scored regions in C1 and I1 of three patients are displayed in Fig. 4. Interestingly, plasma B-cells were the one cell type that was found almost ubiquitously in the biopsied tissue of our cohort of RUTI patients (Fig. 4a, inset; Table S5), suggesting an active humoral adaptive immune response in the chronically inflamed bladder tissue. Loose lymphoid aggregates and edema were found to be present in both C1 and I1 biopsies from PNK010 (Fig. 4a, black and blue arrows, respectively). Denuded epithelium (gray arrow), which is a marker of severe acute inflammation, was only detected in the I1 biopsy and not the C1 biopsy of PNK010. Partial urothelial exfoliation was noted in biopsies from eight patients.

Another change in urothelial architecture, squamous metaplasia, was observed in the urothelium of five patients (Table S5) including PNK011 (Fig. 4b, red arrow). Squamous metaplasia describes the conversion of mature, non-squamous epithelial cells to cells with a squamous morphology. This cellular conversion is a common response to long-standing mucosal irritation. In the I1 biopsy of PNK011, this cellular conversion was coupled with an expansion of the transitional and squamous layers of the bladder urothelium, as well as considerable edema (Fig. 4b, blue arrow). We attribute these changes in urothelial morphology to the chronic nature of the cystitis associated with RUTI in this patient cohort. The urothelium of the C1 biopsy from PNK013 demonstrated no acute inflammation, edema (blue arrow), and only mild signs of chronic inflammation, and was decorated by intact umbrella cells (Fig. 4c, green arrow). The I1 biopsy from PNK013, however, harbored a large, well-formed lymphoid aggregate with a germinal center indicative of a strong adaptive immune response (Fig. 4c, yellow arrow), but only exhibited a mild acute inflammatory response and limited epithelial shedding (gray arrow) (Fig. 4c, Table S5). Our pathological analysis of RUTI patient bladder biopsies suggests that cellular mediators of both acute and chronic inflammation play important roles in shaping the immunological landscape of the RUTI patient bladder.

Discussion

This work provides the first systematic analysis of patient bladder biopsies from postmenopausal women with RUTI. Our understanding of RUTI disease in postmenopausal and elderly women is severely limited, but these populations are often the most difficult to treat. Declining kidney function, age-associated immune decline, and comorbidities not only put older women at increased risk of RUTI but also complicate antibiotic treatment [7]. Furthermore, the rate of antibiotic resistance in uropathogens is high and the long-term prophylactic antibiotic therapy often prescribed to manage RUTI likely contributes to the rapid evolution of antibiotic resistance in uropathogens [35]. In order to devise more effective treatment strategies, the pathogenic mechanisms and inflammatory responses relevant to RUTI in postmenopausal and elderly women must be understood. The IBC-QIR model has been proposed to explain how uropathogens can persist in the bladder and survive antibiotic treatment and host immune responses. QIRs are especially relevant to RUTI as they are thought to be the seed from which infection re-initiates [20,22]. A recent whole-genome sequencing study of *E. coli* isolated from the urine of young women with RUTI found that same-strain RUTI did occur in some, but not all cases [36]. Another study found filamentous bacteria and exfoliated epithelial cells containing intracellular UPEC in urine samples from young, pre-menopausal women with acute, uncomplicated cystitis [37]. Additional urinalysis studies suggest that other UTI pathogens are able to form similar intracellular reservoirs, and a study from 1985 reported the isolation of bacteria from bladder biopsies from women with acute UTI, but the authors did not eliminate extracellular bacteria in their experimental approach [38–40]. Our study therefore provides the first direct evidence that bacterial communities that are qualitatively similar to IBCs and QIRs in size and that infections with various epithelial depths exist in the bladder wall of human RUTI patients.

We show that diverse bacterial species, in additional to canonical uropathogens like *E. coli*, can be cultured from the bladder wall of postmenopausal RUTI patients. In line with these findings, a database review of urine cultures from a cohort of 116 postmenopausal patients with RUTI found an incidence of 57% *E. coli*, 17% *E. faecalis*, 11% *K. pneumoniae*, and 15% other bacteria including *Staphylococcus epidermidis*, *Streptococcus agalactiae*, and *P. mirabilis,* suggesting that *E. coli* infections were much less common in postmenopausal RUTI patients than premenopausal RUTI patients [12]. Another important finding of our study was that in many cases, intracellular or intratissue bacteria were detected in biopsies by FISH from patients where no bacteria were cultured from bladder tissue or urine. It is possible that these bacteria are just *E. coli* that have entered a *viable but not culturable* state, but an intriguing hypothesis is that these intracellular bacteria may be anaerobic or otherwise not culturable by standard aerobic techniques. The anaerobic bacterium *Gardnerella vaginalis,* for example, is common member of the vaginal microbiota that

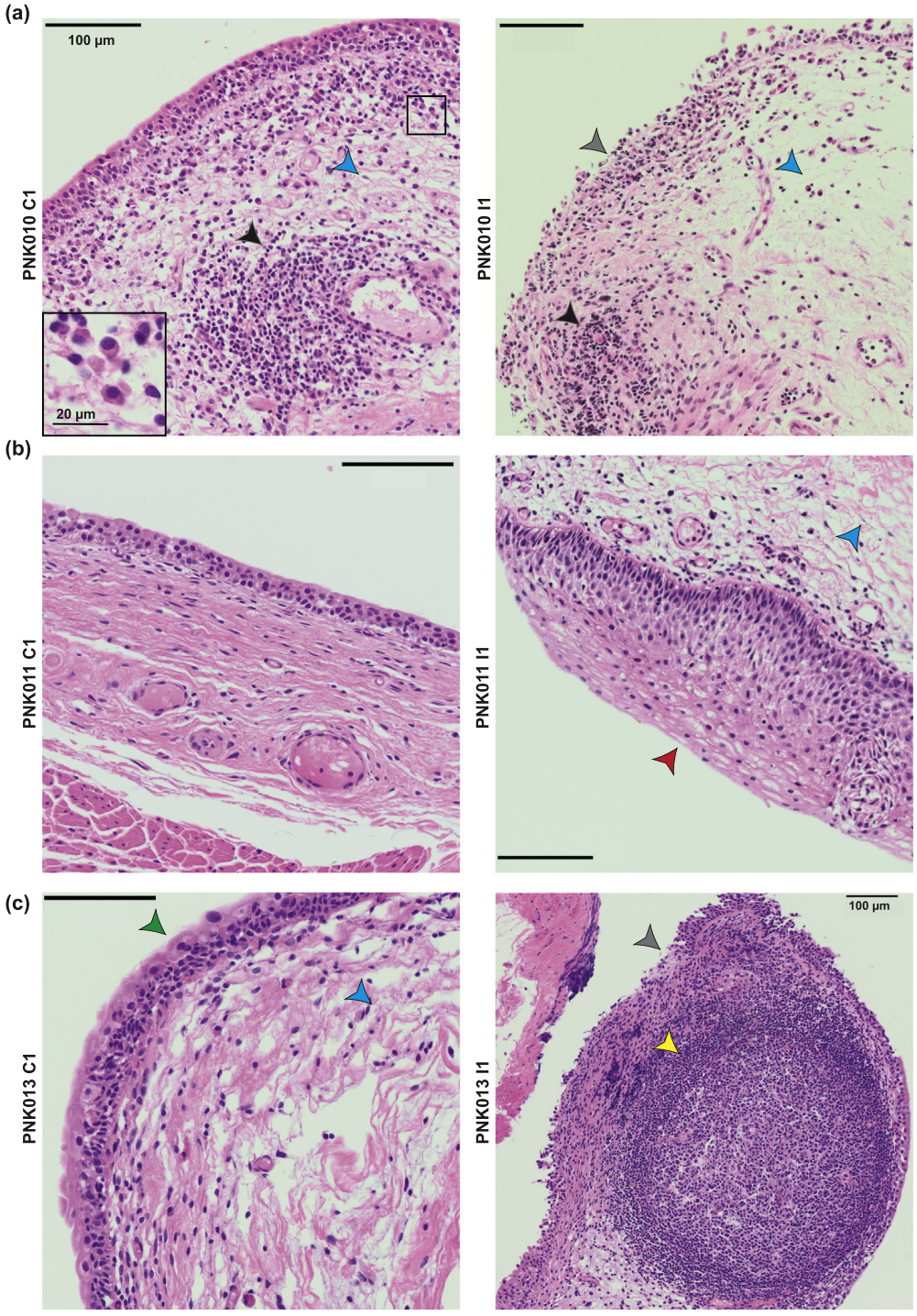

Fig. 4. Inflammation and epithelial remodeling in human RUTI. Representative micrographs of H&E-stained sections of bladder biopsies from visibly normal (C1) and visibly infected (I1) regions from three patients, (a) PNK010, (b) PNK011, and (c) PNK013. Relevant histopathological findings are indicated by colored arrows. Black, lymphoid aggregate; blue, edema; gray, denuded epithelium; red, squamous metaplasia; green, normal umbrella cells; yellow, well-formed lymphoid aggregate with germinal center. Inset in the left side of panel a depicts mesenchymal plasma B-cells. All scale bars are 100 μm except inset (25 μm).

has been found to be present in urine [41]. Women colonized with *G. vaginalis* were found to be at higher risk for UTI than women where *Lactobacillus* spp. were the predominant members of the vaginal microbiota [42]. However, a recent study in mice suggests that *G. vaginalis* colonization may predispose women to

UTI by triggering urothelial exfoliation and reactivation of *E. coli* QIRs and not direct formation of persistent communities themselves [43]. Future work will focus on determining which bacterial species form the intracellular and intra-tissue communities observed within the RUTI patient bladder wall.

The host immune response has been proposed to play a vital role in predisposing women to recurrent UTI. In mouse models, UTI and even RUTI-associated inflammation has primarily been defined as acute, involving a strong neutrophil response and an attenuated adaptive immune response [24,26]. Permanent remodeling of the urothelium include changes in umbrella cell size and function, as well as a marked expansion of the transitional epithelium, was found to occur in mice that were sensitized to recurrent infection [23]. We did observe significant changes in the urothelial architecture of biopsies from both visibly inflamed and non-inflamed regions of the RUTI patient bladder. Interestingly, we did not only observe an expansion of transitional urothelium but also observed squamous metaplasia in several patient biopsies. These observations may be due to the difference in the timeline of RUTI in mice and human patients, since our patient cohort has been experiencing RUTI and the associated bladder inflammation for years, whereas mice used in RUTI models are only monitored for months and their infection is usually completely resolved by antibiotic therapy between recurrent episodes [23].

In this work, we also established that the cellular adaptive immune response is active in human RUTI patients. This was an interesting finding because work in mice has suggested that the cellular adaptive immunity is not properly activated during RUTI and is actually subverted by bladder-resident macrophages [26]. We not only found large lymphoid aggregates in the tissue of many RUTI patients, but also mesenchymal and urothelial plasma B-cells in biopsies from the majority of patients, suggesting that the immunological landscape of UTI in humans may differ from that in mice. Future studies will explore the co-localization of bacteria and immune cells within the bladder wall of these patients. Other aspects of the host environment are likely also contribute to the increased susceptibility to RUTI in postmenopausal women. Previous pregnancy, for example, may also be an important risk factor for RUTI in postmenopausal women. It has been shown in mouse models that increased age combined with multiple previous pregnancies results in heightened susceptibility to chronic UPEC infection [44]. Previous pregnancy, hormonal changes, and aging likely cause alterations to the genitourinary tract that make it a distinct environment from the premenopausal genitourinary tract. Our findings represent a critical step in the understanding of RUTI in postmenopausal women, but further work is needed to better understand the role of intra-tissue bacteria, inflammation, and the adaptive immune response in human RUTI patients.

Methods

Patient recruitment

Following institutional review board (UTSW STU 032016-006, UTSW STU 082010-016, UTD MR 17-120) approval, postmenopausal female patients undergoing CFT were recruited from the urology practice of Dr. Philippe Zimmern at the University of Texas Southwestern Medical Center. Female patients with markers of complicated RUTI including post-void residual >100 mL by bladder scan, $>$ stage 2 bladder prolapse, unstable diabetes, pelvic procedure for incontinence, or pelvic organ prolapse less than 6 months prior, ongoing chemotherapy, renal insufficiency, use of indwelling catheters, intermittent catheterization, neurogenic bladder, no cystitis found on cystoscopy, or any upper urinary tract anomaly explaining RUTI were excluded from the study.

Sample collection

Bladder biopsies and urine were obtained from consenting female patients that met study criteria for antibiotic-refractory RUTIs and presented with office-based evidence of chronic cystitis on flexible cystoscopy. All samples were obtained in the operating room (OR) under anesthesia. Pre-procedure (PRE) urine was immediately stored on ice and processed less than 2 h following collection. Biopsies used for culture were transferred directly into $1\times$ sterile PBS $+$ 100 μg/mL Pn/Gm in the OR. Biopsies used for histological and FISH analysis were placed immediately into 4% paraformaldehyde in the OR.

Biopsy and urine culture

PRE urine (125 μL) was plated on both Brain Heart Infusion (BHI) and Chromagar Orientation (CHR) plates and incubated at 37 °C. CHR plates are specifically designed for the chromogenic screening of a wide variety of common uropathogens and allowed the easy identification of diverse species on a single plate by colony color [45]. BHI was used as a rich growth media due to its ability to support the growth of fastidious bacteria [46]. For CFU enumeration, urine was serially diluted and spotted on BHI agar. Biopsies were washed three times in sterile $1\times$ PBS, and then homogenized with a sterile pestle and lysed in 500 μL of 0.5% Triton X-100 for 15 min. Sterile $1\times$ PBS (500 μL) was added to the lysates and vortexed, and then 125 500 μL was plated on BHI and CHR plates and incubated at 37 °C.

16S rRNA gene sequencing

Colony PCR with universal primers to amplify the 16S rRNA gene (8F and 1492R) [47] was performed

on well-isolated colonies grown overnight in BHI. PCR products were gel-purified and Sanger-sequenced (Genewiz). Forward and reverse reads were assembled to generate a ~1.5-kb contig for each strain. Species identification was performed by querying the nr/nt database using the megablast algorithm for sequences with >99% identity.

Antibiotic susceptibility testing

Antibiotic susceptibility testing of urine and tissue isolates was performed by the Clements University Hospital Clinical Laboratory using the MicroScan Walkaway System (Beckman Coulter) following standard protocols.

Histopathology

Biopsies used for histological and FISH analysis were allowed to fix in sterile 4% para-formaldehyde for 6 h at room temperature and then stored in 1× sterile PBS at 4 °C. Biopsies were then paraffin embedded and longitudinally sectioned by the UTSW Molecular Pathology Core with sterile solutions and equipment. Sections from each biopsy were stained with hematoxylin and eosin and scored blind by a pathologist.

FISH

All staining steps were performed in aseptic conditions. An adapted protocol from Vaishnava *et al*. [32] was used for FISH. Biopsy sections were deparaffinized in xylene and rehydrated in ethanol and sterile water. Tissues were incubated with 10 nM Alexa-488 conjugated probe in filter-sterilized 20 mM Tris−HCl (pH 7.4), 0.9 M NaCl, and 0.1% SDS overnight at 50 °C, washed and stained with 1 μg/mL Hoechst, 5 μg/mL Alexa555-WGA, and 13.2 nM Alexa555-phalloidin, then washed in sterile 1× PBS and mounted. Confocal microscopy was performed on a Zeiss LSM800 with a 63× objective. Images were processed and analyzed with ImageJ.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2019.04.008.

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Abbreviations used:
UTI, urinary tract infection; RUTI, recurrent urinary tract infection; CFT, cystoscopy with fulguration of trigonitis; UPEC, Uropathogenic *Escherichia coli*; IBC, intracellular bacterial community; QIR, quiescent intracellular reservoir; FISH, fluorescence *in situ* hybridization; Pn, penicillin; Gm, gentamicin.

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