Gut microbiome correlates of recurrent urinary tract infection: a longitudinal, multi-center study

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Summary

Background Urinary tract infections (UTI) affect approximately 250 million people annually worldwide. Patients often experience a cycle of antimicrobial treatment and recurrent UTI (rUTI) that is thought to be facilitated by a gut reservoir of uropathogenic *Escherichia coli* (UPEC).

Methods 125 patients with UTI caused by an antibiotic-resistant organism (ARO) were enrolled from July 2016 to May 2019 in a longitudinal, multi-center cohort study. Multivariate statistical models were used to assess the relationship between uropathogen colonization and recurrent UTI (rUTI), controlling for clinical characteristics. 644 stool samples and 895 UPEC isolates were interrogated for taxonomic composition, antimicrobial resistance genes, and phenotypic resistance. Cohort UTI gut microbiome profiles were compared against published healthy and UTI reference microbiomes, as well as assessed within-cohort for timepoint- and recurrence-specific differences.

Findings Risk of rUTI was not independently associated with clinical characteristics. The UTI gut microbiome was distinct from healthy reference microbiomes in both taxonomic composition and antimicrobial resistance gene (ARG) burden, with 11 differentially abundant taxa at the genus level. rUTI and non-rUTI gut microbiomes in the cohort did not generally differ, but gut microbiomes from urinary tract colonized patients were elevated in *E. coli* abundance 7–14 days post-antimicrobial treatment. Corresponding UPEC gut isolates from urinary tract colonizing lineages showed elevated phenotypic resistance against 11 of 23 tested drugs compared to non-colonizing lineages.

Interpretation The gut microbiome is implicated in UPEC urinary tract colonization during rUTI, serving as an ARG-enriched reservoir for UPEC. UPEC can asymptomatically colonize the gut and urinary tract, and post-antimicrobial blooms of gut *E. coli* among urinary tract colonized patients suggest that cross-habitat migration of UPEC is an important mechanism of rUTI. Thus, treatment duration and UPEC populations in both the urinary and gastrointestinal tract should be considered in treating rUTI and developing novel therapeutics.

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Keywords: Gut microbiome; Recurrent urinary tract infection; Antimicrobial resistance; *Escherichia coli*

Research in context

Evidence before this study

We searched PubMed for the terms "recurrent urinary tract infection" (rUTI) AND "microbiome" AND "longitudinal" with no language restrictions from database inception to December 19, 2023. Of the 6 search results, only 1 study from 2022 sequenced the gut microbiome, comparing 15 women with rUTI with 16 healthy controls. Among the search results was also a previous study from our group demonstrating clonal persistence of uropathogenic isolates in the intestinal and urinary tracts. To our knowledge, this is the first study considering both clinical and microbiome characteristics of patients with MDRO *E. coli* rUTI against those with UTI but no recurrence during the follow-up period.

Added value of this study

This study expands upon a previously published UTI patient cohort, analyzing the largest cohort of UTI patient microbiomes to date, with longitudinal gut microbiome data and matched clinical data from 125 patients. Gut microbiomes from rUTI and UTI patients did not exhibit global differences, but asymptomatic colonization of the urinary tract by uropathogenic *Escherichia coli* (UPEC) corresponded to elevated gut *E. coli* abundance, as well as increased phenotypic resistance against 11 of 23 tested antimicrobials.

Implications of all the available evidence

Asymptomatic colonization of the urinary tract is linked to differential gut microbiome composition, suggesting in-host UPEC dynamics are intertwined with the commensal community. These findings serve to inform future efforts to elucidate rUTI mechanism and develop therapeutics to target rUTI at the source.

Introduction

Urinary tract infections (UTIs) are estimated to affect 250 million people worldwide each year.[1] In the United States (US) alone, 13.7% of men and 60% of women experience a UTI in their lifetime,[2,3] and 24% of women with UTI experience recurrent UTI (rUTI) within 6 months of the initial episode.[4] As UTIs are typically treated with antimicrobials, the cycle of treatment and recurrence is fertile ground for selection of antimicrobial resistance (AR).[5] Uropathogenic *E. coli* (UPEC) are the most common causative agents of UTI,[6] and comparative genomic analyses of UPEC have established that the cycle of recurrence is fueled by at least three independent pathways: urinary persistence, reinfection from external sources, and gastrointestinal colonization.[7–10] The gut in particular is a known reservoir for UPEC, from which multiple episodes of UTI can be seeded.[7–9]

In healthy individuals, commensal microbiota populating the gut can provide colonization resistance against pathogenic Enterobacterales through competitive exclusion or by modulating host immunity.[10] A disrupted gut microbiome state has been implicated in a number of chronic and recurrent conditions, including *Clostridioides difficile* infection (CDI)[11] and inflammatory bowel disease (IBD).[12] Similarly, the history of repeated antimicrobial exposures in rUTI may render patients more susceptible to colonization with UPEC.[9] One recent study comparing the gut microbiomes of 15 women with a history of rUTI and 16 healthy controls reported depleted richness in the gut microbiome in women with rUTI, including depleted richness and reduced abundance of butyrate producers.[13] However, our understanding of UPEC's role in the gut microbiome and which factors drive some UTI patients towards recurrence is incomplete. The purpose of this 125-patient, multicenter, prospective cohort study was to investigate the relationship between urinary tract colonization, gut microbiota, and rUTI, controlling for clinical characteristics.

Methods

Study context

A subset of this cohort was originally published in a pilot study of 14 patients in Thänert et al., 2019.[14] This current study utilizes the same study population as Thänert and Choi et al., 2022.[15] Whereas the previous study assessed persistence of *E. coli* lineages in the urinary tract and gut, here we expand upon the prior dataset by investigating an accompanying set of 644 stool microbiome samples and patient-level clinical metadata.

Study population

Patients for this prospective, multi-center cohort study were recruited between July 2016 and May 2019 among patients with positive clinical urine cultures at Barnes-Jewish Hospital/Washington University in St. Louis (WU), St. Louis, Missouri; Duke University Hospital (DK), Durham, North Carolina; the Hospital of the University of Pennsylvania (PN), Philadelphia, Pennsylvania; and Rush University Medical Center (RH), Chicago, Illinois.

Inclusion/exclusion criteria

Patients with a symptomatic UTI diagnosed and treated by a physician and a urine culture that yielded Enterobacterales with one of the following resistances were included in the current analysis: (1) resistance to ciprofloxacin or levofloxacin, (2) resistance to any third generation cephalosporin, (3) resistance to ertapenem and susceptible to meropenem, imipenem, and/or doripenem, (4) resistance to >2 of the following antimicrobial classes: carbapenems, aminoglycosides, fluoroquinolones, fourth generation cephalosporins, piperacillin/tazobactam, or (5) identification of any of the following resistance mechanisms: ESBL, CRE, KPC, NDM-1, OXA-48, IMP, IMP-1, or VIM.

Patients were excluded if they had any of the following conditions: >1 organism in their urine, recurrent CDI, intra-abdominal devices, absolute neutrophil count [ANC] <500 mm^3, intestinal mucosal disruption, unlikely to survive 6 months, pregnancy or unwilling/unable to use contraception, short gut syndrome, intestinal motility medication use, irritable bowel disease, recent abdominal surgery, active typhlitis or diverticulitis, current gastrointestinal graft-versus-host disease, HIV without antiretroviral therapy, CD4 <200 mm^3, peritoneal dialysis, cirrhosis with ascites, active intra-abdominal malignancy, chronic indwelling foley or suprapubic catheter, chronic ileal conduit, active hepatitis B or C, ureteral stent, or active kidney stone. Patients were also excluded if their urine culture was considered to have insufficient growth based on clinical standards (as determined by the clinical laboratory). Using this criteria, a total of 586 patients were screened as highly eligible.

Enrollment

Eligible patients were contacted by study personnel by phone (if outpatient) or in person (if hospitalized) to verify that all inclusion/exclusion criteria were met. A total of 187 patients were initially enrolled in this study. Once a patient was enrolled, study personnel interviewed the patient regarding their UTI symptoms, UTI antimicrobial treatment, and medical history. If available, study personnel also collected remnant urine from the patient's diagnostic urine culture from the clinical microbiology laboratory. 62 patients dropped out from the study after enrollment and during the follow-up period, resulting in a final study population of 125 patients.

Ethics statement

Written, informed consent was obtained from all patients. This study was approved by the Washington University Human Research Protection Office as the single IRB (#20161019). Local IRB approvals were obtained as necessary (Rush University Medical Center #15122910, University of Pennsylvania Institutional Review Board #812302, and Duke Health Institutional Review Board #281214).

Episode and outcome definitions

The first UTI episode per patient was defined as starting on the date of study enrollment. UTI recurrence (rUTI) was defined as the diagnosis of a subsequent symptomatic UTI that required antimicrobial treatment during the six-month follow-up period with any uropathogen. All UTI diagnosis and treatment decisions were made by the patient's primary treatment provider. The recurrence date was assigned as the date of first symptom onset if known; otherwise, the antimicrobial treatment start date was used. If a patient continued in the study, the recurrence date served as both the end of follow-up for the episode and the start date for a new UTI episode. From episode 1 enrollment, a patient could continue in the study for to up to three total UTI episodes; patients with a fourth UTI were censored at that time. Patients who did not develop a rUTI were followed for up to 6 months.

Specimen and data collection

Patients submitted stool and urine specimens to the study team at enrollment (Sample 1), the end of UTI antimicrobial treatment (S2), and days 3 (S3), 7 (S4), 14 (S5), 30 (S6), 60 (S7), 90 (S8), 120 (S9), 150 (S10), and 180 (S11) post-antimicrobial treatment. If a patient had a recurrence and chose to continue in the study, the stool and urine specimen collection schedule restarted as a new episode (E1, E2, E3).

At each collection point, patients were provided with supplies for collecting their stool and urine, along with questionnaires about UTI symptoms, medications received, and changes in medical history. Stool/urine

specimens and questionnaires were shipped to the study team by courier. Upon arrival in the laboratory, samples were immediately processed for microbiologic culture or frozen at –80 °C. Stool and urine samples collected at sampling points S1, S2, S4, S6, and S11 were selectively cultured to assess uropathogen persistence. If a patient did not submit a specimen at a sampling point, the sample collected at the next closest time point was selected for analysis.

Selective culture

Approximately 1 g of stool samples collected at enrollment and on days 0, 7, 30, and 180 post-antimicrobial treatment (pAT) were supplemented with an equal amount (wt/vol) of phosphate-buffered saline (PBS) and vortexed to homogenize the samples. Ten 10-fold serial dilutions were prepared in PBS, and 10 µL of each of the first 10 dilutions was streaked onto selective agar (Hardy Diagnostics, Santa Maria, CA, USA) specific to each patient's identified ARO using a 10 µL calibrated loop. MacConkey (MAC) agar supplemented with ciprofloxacin (10 µg/ml) was used for ciprofloxacin-resistant Enterobacteriaceae, while ESBL-producing Enterobacteriaceae were cultured on Hardy Diagnostics ESBL agar and MAC agar supplemented with cefotaxime (1 µg/ml). Isolate species was confirmed using MALDI-TOF MS (VITEK MS, bioMérieux, Durham, NC, USA). Single colonies were diluted in TSB/glycerol and stored at –80 °C for later analysis.

DNA extraction, sequencing and quality filtering

Metagenomic DNA for stool microbiome profiling was extracted from ~100 mg of frozen stool using the DNeasy PowerSoil kit (Qiagen, Germantown, MD, USA). Sequencing libraries from fecal metagenomic DNA were prepared using the Nextera kit (Illumina, San Diego, CA, USA). Libraries were pooled and sequenced (2 × 150 bp) to a depth of ~5 million reads (fecal metagenomes) on the NextSeq 500 HighOutput platform (Illumina, San Diego, CA, USA). The resulting reads were trimmed of adapters using Trimmomatic v.36 (parameters: LEADING:10 TRAILING:10 SLIDINGWINDOW:4:15 MINLEN:60) and depleted of human read contamination using DeconSeq v.4.3 (default parameters).[16,17]

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) of cultured isolates was performed on Mueller Hinton agar (Hardy Diagnostics, Santa Maria, CA, USA) using Kirby Bauer disk diffusion with antimicrobial disks purchased from Hardy Diagnostics (Santa Maria, CA, USA) and Becton Dickinson (Franklin Lakes, NJ, USA). Results were interpreted according to Clinical & Laboratory Standards Institute guidelines.[18] Firth's Bias-Reduced Logistic Regression was conducted for each drug to test for association between resistance and urinary tract colonization using logistf package[19] in R v3.6.3.[20] To this end, we counted the number of resistant and non-resistant isolates between urinary tract colonizing and non-colonizing lineages of *E. coli*, where intermediate isolates were grouped together with susceptible isolates as 'non-resistant.' We then modeled drug resistance ~ urinary tract colonization status as binary vectors. To calculate AST scores, the AST data were converted into a numeric matrix (0: susceptible, 0.5: intermediate, 1: resistant) and summed for each isolate.

UPEC colonization

UPEC colonization definitions were retained from an earlier publication from this cohort.[15] Briefly, UTI episodes were categorized as colonized by UPEC if (1) the same *E. coli* lineage was recovered from a specimen type (stool/urine) at >1 asymptomatic sample, or (2) if all isolates recovered from a specimen type (stool/urine) from a UTI episode belonged to the same *E. coli* lineage. Ultimately, colonization for a UTI episode was dichotomized for analysis to represent urinary tract and gastrointestinal colonization any time during the follow-up period before the next recurrence or censor date. Colonization status was re-set at the start of any subsequent UTI episodes. For the purposes of the study, we define UPEC as isolates recovered at asymptomatic time points belonging to the same lineage as a diagnostic urine isolate (DxU).

Statistical analysis

We used univariate and multivariable Prentice, Williams, and Peterson (PWP) total time model—a conditional model extension of the Cox proportional hazards model that models the full time course of recurrent events—to explore the role of uropathogen colonization and risk of rUTI, controlling for clinical characteristics.[21,22] Most clinical variables were binary. Variables with multiple categories were collapsed using clinical knowledge and logical groupings to avoid small cell sizes (e.g., race). For BMI, we utilized CDC BMI categories for normal weight, overweight, and obesity. The time zero (origin) for each patient was assigned as the study enrollment date, which also served as the start date for episode 1. The end date for each episode was the recurrence date if a rUTI occurred or the censor date if the patient did not have a rUTI. The start date for episodes 2 and 3, if the patient continued in the study, was the rUTI date that ended the previous episode. Potential risk factors for rUTI were collected from the baseline questionnaire. The proportional hazards assumption was assessed and confirmed for all potential variables via visualization of the negative logarithm of estimated survivor functions plots for each covariate. Data management was performed using REDCap and SPSS v27 (IBM Corp., Armonk, NY), and statistical analysis was performed using SAS version 9.4 (SAS Institute Inc., Cary, NC).

Microbiome analysis

To assess differences in gut microbiota between participants with a history of UTI compared to a healthy population, we downloaded two publicly-available metagenomic datasets from recent studies in the US: microbiomes from 20 healthy adults (PRJNA664754; "HH"; 50% female, median age 29) as well as 31 microbiomes from a rUTI study ("UMB") comprising 15 rUTI (>2 episodes of UTI in past 12 months) and 16 healthy participants (<2 UTIs in lifetime; PRJNA400628; 100% female, median age 37; Supplemental Table S1). The first available metagenomic stool sample from every individual was used. Both datasets featured sequencing depth >2.5 million reads per sample, and the HH cohort utilized identical metagenomic DNA extraction and sequencing techniques as this study.

Paired-end metagenomic reads from all cohorts were used to access sample-specific microbial taxa relative abundance using MetaPhlAn3 v.3.1.0 (default parameters).[23] Average taxonomic profiles for each patient were also generated by averaging the relative abundances of each taxon at the species level. This process was also repeated to generate average taxonomic profiles per patient at specific timepoints. Taxa were filtered for 10% prevalence prior to each analysis. Resistance gene abundance was determined using ShortBRED v.0.9.4[24] using marker sequences built on the CARD and NCBI AMR databases.

Statistical analysis and visualization of gut microbiome data were conducted in R v.3.6.3.[20] α- and β-microbiota diversity were calculated using vegan v2.5.7.[25] Repeat measures permutational analysis of variance (PERMANOVA) was implemented using the adonis function. Covariates are added sequentially in adonis, such that potential confounding factors can be prioritized in the model when expected to account for a great amount of variation. For repeat measures, Patient ID was included as the first PERMANOVA term. In cross-cohort comparisons, a unique study ID was assigned per cohort and included as the first PERMANOVA term. For within-cohort comparisons, age (18–64; 65–79; ≥80) and UTI treatment antimicrobial were included as categorical variables. We additionally conducted a cross-cohort comparison using an age and sex-matched subset of the current cohort (20 samples from female patients with median age 31, IQR 25–37), to eliminate potential confounding factors. Linear mixed-effects models (LMEs) were implemented at the species level using the MaAsLin2 package via arcsine square root transformation.[25] LMEs included study ID as a random effect in cross-cohort comparisons, and age and treatment drug as categorical random effects in within-cohort comparisons. The phyloseq[26] package was used to calculate pairwise Bray–Curtis distance between samples and conduct ordination via principal coordinates analysis (PCoA) and canonical analysis of principal coordinates (CAP). *P*-values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg (BH) where appropriate. Visualizations were created using ggplot2[27] and ggpubr.[28]

Role of the funding source

The funders of this study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. J.C., E.R.D., J.H.K, and G.D. have full access to all the data in the study and have final responsibility for the decision of submission to the journal for publication.

Results

A total of 125 patients were enrolled in the study from the four participating sites (Table 1, Fig. 1A). Forty-seven (37.6%) patients experienced rUTI within 6 months. 12/38 (31.6%) patients who continued in the study after their first recurrence experienced a second recurrence, and 7/12 (58.3%) of those who continued in the study after their second recurrence experienced a third recurrence. The median and interquartile range (IQR) of follow-up time for the 175 episodes was 155 days (IQR, 35, 190). The reasons for censoring were as follows: 66 (38%) ended in a rUTI, 29 (17%) ended due to patient withdrawal from the study, and 80 (46%) completed the follow up period.

Most patients were female (93.6%) with a median age of 58 years (interquartile range 42–71). 92.8% of first UTI episodes were caused by *E. coli* (Table 2). A minority of patients (5%) were hospitalized at the time of enrollment. The most common symptoms of UTI episodes were pain or burning during urination and cloudy urine (>40% of patients experienced each of these symptoms). The most common antimicrobials used to treat UTI episodes were nitrofurantoin (44.6%) and cephalosporin or a penicillin (30.3%).

Recurrence not associated with underlying clinical characteristics

The rates of UTI recurrence by patient factors, infection history, UTI history, baseline medications, and colonization status are reported in Supplemental Table S2. No clinical characteristics were independently associated with rUTI at the 0.05 threshold in the total cohort or when restricted to females (Table 3).

The gut microbiome in UTI patients is distinct from that of healthy individuals

To characterize the gut microbiome, 644 stool samples from 106 patients with available stool were sequenced (Fig. 1B). Forty-three (40.6%) of these patients experienced 45 episodes of rUTI during the study period, and 63 did not (59.4%; non-rUTI). In total, 331 rUTI samples and 313 non-rUTI stool samples were subject to whole metagenome sequencing. The enrollment samples from this cohort (E1-S1; n = 96) were grouped

Factor	Value	Total cohort (n = 125) n (%)	Cohort with recurrence (n = 47) n (%)	Cohort without recurrence (n = 78) n (%)
Demographics				
Female		117 (93.6)	44 (93.6)	73 (93.6)
Age (years), median (interquartile range)		58 (42, 71)	59 (42, 72)	58 (41, 69)
Race	White	73 (58.4)	27 (57.4)	46 (59.0)
	African-American	47 (37.6)	18 (38.3)	29 (37.2)
	Other	5 (4.0)	2 (4.3)	3 (3.8)
Hispanic		5 (4.0)	2 (4.3)	3 (3.8)
Study site	1	41 (32.8)	16 (34.0)	25 (32.1)
	2	23 (18.4)	6 (12.8)	17 (21.8)
	3	13 (10.4)	4 (8.5)	9 (11.5)
	4	48 (38.4)	21 (44.7)	27 (34.6)
Comorbidities				
Body mass index (kg/m^2)	Normal/underweight (<18.5–24.9)	32 (25.6)	11 (23.4)	21 (26.9)
	Overweight (25.0–29.9)	39 (31.2)	16 (34.0)	23 (29.5)
	Obese (≥30.0)	54 (43.2)	20 (42.6)	34 (43.6)
Solid tumor		22 (17.6)	9 (19.1)	13 (16.7)
Cardiovascular disease (myocardial infarction, congestive heart failure, and peripheral vascular disease)		22 (17.6)	7 (14.9)	15 (19.2)
Cerebrovascular disease		2 (1.6)	1 (2.1)	1 (1.3)
Chronic obstructive pulmonary disease		2 (1.6)	1 (2.1)	1 (1.3)
Chronic renal failure		8 (6.4)	4 (8.5)	4 (5.1)
Diabetes mellitus		32 (25.6)	11 (23.4)	21 (26.9)
Leukemia or lymphoma		2 (1.6)	1 (2.1)	1 (1.3)
Other comorbidity[c]		59 (47.2)	20 (42.6)	39 (50.0)
Rheumatologic disease		10 (8.0)	4 (8.5)	6 (7.7)

[a]For patients with >1 UTI episode, information from the first episode is reported. [b]Of 125 patients, 47 (37.6%) initial episodes ended in recurrent UTI (rUTI). Of 47 patients with rUTI, 38 patients continued in the study and 12/38 (31.6%) episodes ended in had another rUTI. All 12 patients continued in the study and 7/12 (58.3%) had a rUTI during their 3rd episode. [c]Any other medical condition noted by participant.

Table 1: **Characteristics of 125 patients with an antibiotic-resistant organism (ARO) urinary tract infection (UTI), overall and by recurrence status.[a,b]**

together with 15 published rUTI samples from the UMB study (See Methods) as "UTI". Microbiome samples from healthy adults (20 HH, 16 UMB) were included as a "Healthy" comparison group.

Species richness was lower among UTI samples compared to healthy controls, though not reaching significance (Kruskal–Wallis, $P = 0.055$ Fig. 2A, Supplemental Table S3). Pairwise microbiome dissimilarity (Bray–Curtis) was measured, and even after accounting for differences among studies (PERMANOVA, $P = 0.001$, Fig. 2B), there were significant differences in species-level microbiota composition between UTI and healthy samples (PERMANOVA, $P = 0.043$, Fig. 2C). These findings were repeated when using an age-matched, all-female subset of the cohorts (20 STL, 10 HH, 31 UMB, PERMANOVA study ID $P = 0.001$, UTI status $P = 0.049$, Supplemental Fig. S1).

Using linear mixed-effect models (MaAsLin2),[25] 11 differentially abundant intestinal taxa were identified at the genus level (False Discovery Rate; FDR < 0.25) between UTI samples and healthy controls, of which 9 were depleted in UTI samples (Fig. 2D, Supplemental Table S4). Genera depleted in UTI samples included *Parasutterella*, *Akkermansia*, and *Bilophila*. The healthy samples were enriched in commensal Firmicutes *Ruminococcus*, *Roseburia*, and *Eubacterium*. Four families and 26 species were also found to be differentially abundant in the analysis (Supplemental Table S4).

We hypothesized the UTI gut microbiome may be enriched for antimicrobial resistance genes (ARGs) compared to the healthy microbiome, due to a history of UTI treatment-related antimicrobial exposure. The abundance of identified ARGs (as measured in units of Reads Per Kilobase of reference sequence per Million sample reads; RPKM) was significantly higher among UTI samples (Kruskal–Wallis, $P = 0.002$, Fig. 2E), but not their richness (Kruskal–Wallis, $P = 0.09$, Fig. 2F) or diversity (Kruskal–Wallis, $P = 0.53$, Supplemental Table S5).

The gut microbiomes of patients with rUTI and those without (non-rUTI) are similar

The gut microbiomes of all 480 samples from each patient's first UTI episode were compared (including S1) to query differences between the rUTI and non-rUTI microbiome. Neither richness (Kruskal–Wallis,

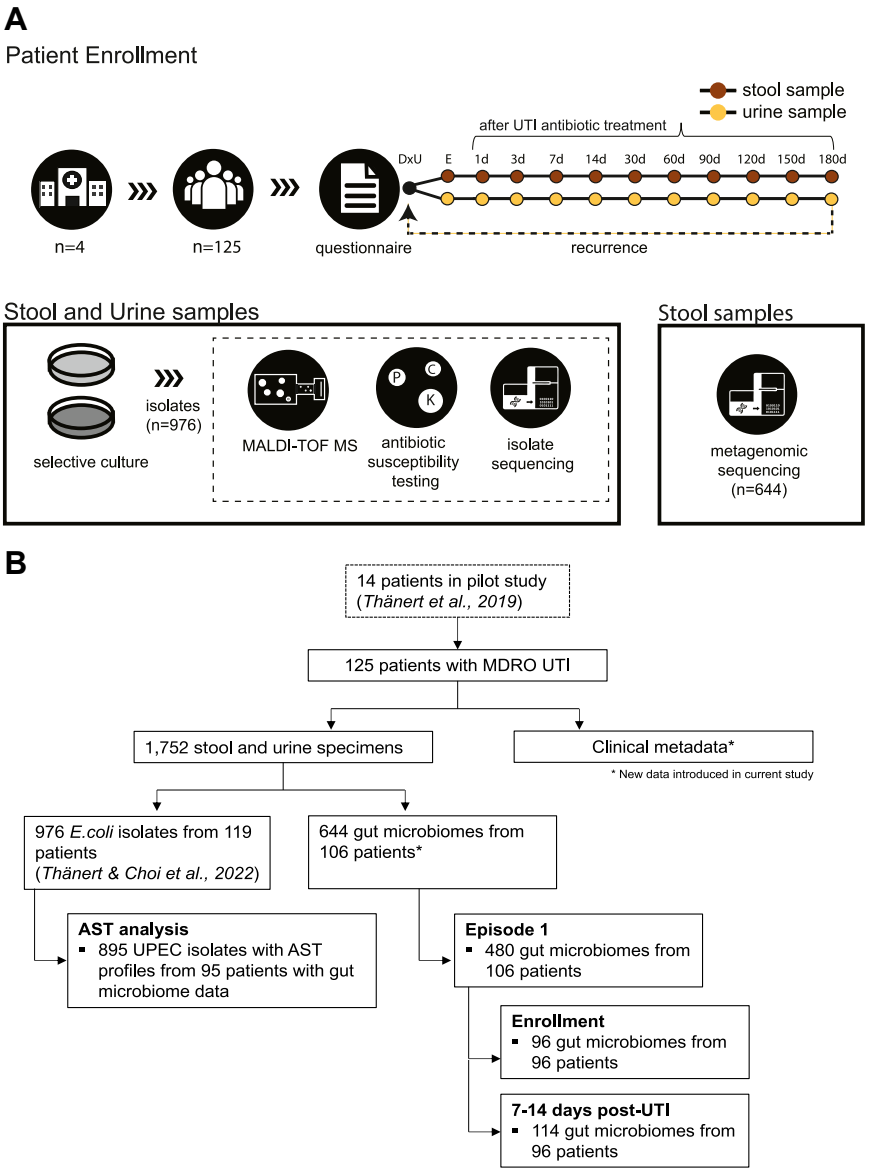

***Fig. 1:* Study overview.** (A) A cohort of 125 patients with UTI were enrolled from four hospital centers in the US. Questionnaires regarding UTI symptoms were collected at time of hospital visit. Stool and urine samples were collected from diagnosis (DxU) to enrollment (E) to 6 months after end of antibiotic treatment for UTI (180 d). Patients experiencing multiple episodes of UTI (rUTI) re-started the follow-up period beginning with another DxU sample. Stool and Urine samples were plated for selective culture, sequenced, and tested for antibiotic susceptibility. 644 stool samples from 106 patients were further subject to metagenomic sequencing. (B) Flow chart illustrating cohort context and samples utilized in previous and current analyses.

P = 0.37) nor Shannon diversity (Kruskal–Wallis, P = 0.24, Supplemental Fig. S2A and B) differed between groups. Patient ID was the greatest source of microbiome variation (PERMANOVA, P = 0.001), but not rUTI status (P > 0.05, Supplemental Fig. S2C). When the analysis was repeated with just one representative taxonomic profile per patient (average relative abundance of each species across all samples per patient; Supplemental Table S6), rUTI status was again not a significant variable explaining microbiome composition (P = 0.35, Supplemental Fig. S2D).

Urinary tract colonized patients have increased gut *E.coli* at 7–14 days post-antimicrobials

Gut microbiome species richness was significantly depleted during and after antibiotic therapy (enrollment, day 3), but increased significantly by days 7–14 post-antimicrobial treatment (Wilcoxon signed-rank test,

Factor	N (%)
UTI antibiotic treatment[a]	
Carbapenem	10 (5.7)
Cephalosporin or a penicillin	53 (30.3)
Doxycycline	4 (2.3)
Nitrofurantoin	78 (44.6)
Quinolone	26 (14.9)
TMP-SMX	28 (16.0)
UTI antibiotic treatment duration >7 days[b]	85 (48.6)
Characteristics of UTI	
Organism, first episode per person (n = 125)	
Citrobacter freundii	1 (0.8)
Escherichia coli	116 (92.8)
Klebsiella pneumoniae	5 (4.0)
Proteus mirabilis	3 (2.4)
UTI symptoms	
Bladder pain	45 (25.7)
Bladder not emptying	48 (27.4)
Blood in urine	21 (12.0)
Burning during urination	84 (48.0)
Chills	29 (16.6)
Cloudy urine	71 (40.6)
Fever	30 (17.1)
Flank pain	49 (28.0)
Other UTI and/or non-specific symptoms	20 (11.4)
Pain during urination	81 (46.3)
Urinary hesitancy	58 (33.1)
Urine odor	67 (38.3)

SMX, sulfamethoxazole; TMP, trimethoprim, UTI, urinary tract infection. [a]Treatment antibiotics are not mutually exclusive, >1 antibiotic was reported for 23 (13.1%) episodes. Thus, the antibiotic treatment includes both empiric antibiotics and any antibiotic changes or additions for definitive treatment or due to an adverse event. [b]Among episodes with >7 days of UTI antibiotic treatment, 21 were treated with >1 antibiotic.

Table 2: **Characteristics of 175 urinary tract infection episodes.**

BH-adjusted $P = 0.018$ for both, Supplemental Fig. S3A). Moreover, antimicrobials differentially impacted microbiome richness at earlier timepoints (Ertapenem and Amoxicillin/Clavulanic acid with lowest richness, Kruskal–Wallis Enrollment $P = 0.003$ and 0–3 days pAT 2 $P = 0.004$, Dunn post-hoc BH-adjusted $P < 0.05$, Supplemental Fig. S3B), but these differences were non-significant by days 7–14 (Kruskal–Wallis $P = 0.0565$, Supplemental Fig. S3B). This observation prompted us to investigate the microbiome at specific timepoints. Urinary tract colonized patients (as defined in the Methods, $n = 33$) had distinct gut microbiomes from non-urinary tract colonized patients ($n = 63$) at days 7–14 post-antimicrobials (PERMANOVA $P = 0.023$ Fig. 3A), even after adjusting for UTI treatment antimicrobial type ($P = 0.044$), age ($P = 0.149$), and sex ($P = 0.018$). The gut microbiome at no other timepoint differed significantly in taxonomic structure by recurrence, urinary tract colonization, or gut colonization.

E. coli and *Paraprevotella xylaniphila* were the only two intestinal taxa significantly enriched in urinary tract colonized patients (MaAsLin2 FDR = 0.15, log2 fold change = 0.28 and 0.19 respectively, Fig. 3B–C, Supplemental Table S4). These cohort-level observations were also quantifiable at the individual scale: Patient WU-16 exhibited a 44-fold increase of intestinal *E. coli* from day 3 to day 7, and a 6-fold increase from day 7 to day 14 (Fig. 3D).

Among the urinary tract colonized patients, 54.5% (18/33) experienced rUTI during the follow-up period. These patients exhibited depleted gut *Bacteroides xylanisolvens* abundance compared to non-rUTI patients, and this was the singular distinguishing taxon observed (MaAsLin2 FDR = 0.05, log2 fold change = –0.38, Fig. 3E).

Intestinal *E. coli* from urinary tract colonized individuals exhibit heightened phenotypic resistance

Gut *E. coli* from urinary tract colonizing lineages were enriched in resistance against 11/23 drugs: ceftriaxone, ceftazidime, cefotetan, cefazolin, ampicillin, TMP-SMX, ampicillin-sulbactam, ciprofloxacin, levofloxacin, aztreonam, and nitrofurantoin (Firth's penalized likelihood logistic regression, BH-adjusted $P < 0.05$, Supplemental Table S7, Fig. 3F). Non-urinary tract colonizing lineages were enriched in resistance against amikacin (BH-adjusted $P < 0.05$). Gut *E. coli* from urinary tract colonizing lineages were elevated in overall

Factor	Value	Univariate HR for rUTI (95% CI) N = 175	Multivariable HR for rUTI (95% CI) N = 175	Multivariable HR for rUTI, among females (95% CI) N = 164
Steroids in 6 months before/at UTI episode start		1.89 (1.08, 3.31)	1.62 (0.91, 2.87)	1.53 (0.85, 2.76)
Any antibiotics in 6 months before/at UTI episode start (other than UTI antibiotic treatment at episode start)		2.03 (0.96, 4.28)	1.80 (0.85, 3.82)	1.72 (0.80, 3.69)
Urinary tract colonization	Not colonized	Ref.	Ref.	Ref.
	Colonized	1.58 (0.94, 2.66)	1.41 (0.83, 2.39)	1.41 (0.82, 2.44)
	Unknown	0.68 (0.26, 1.78)	0.70 (0.27, 1.84)	0.70 (0.27, 1.86)

Table 3: **Univariate and multivariable risk factors for recurrence after urinary tract infection (UTI), clinical model.**

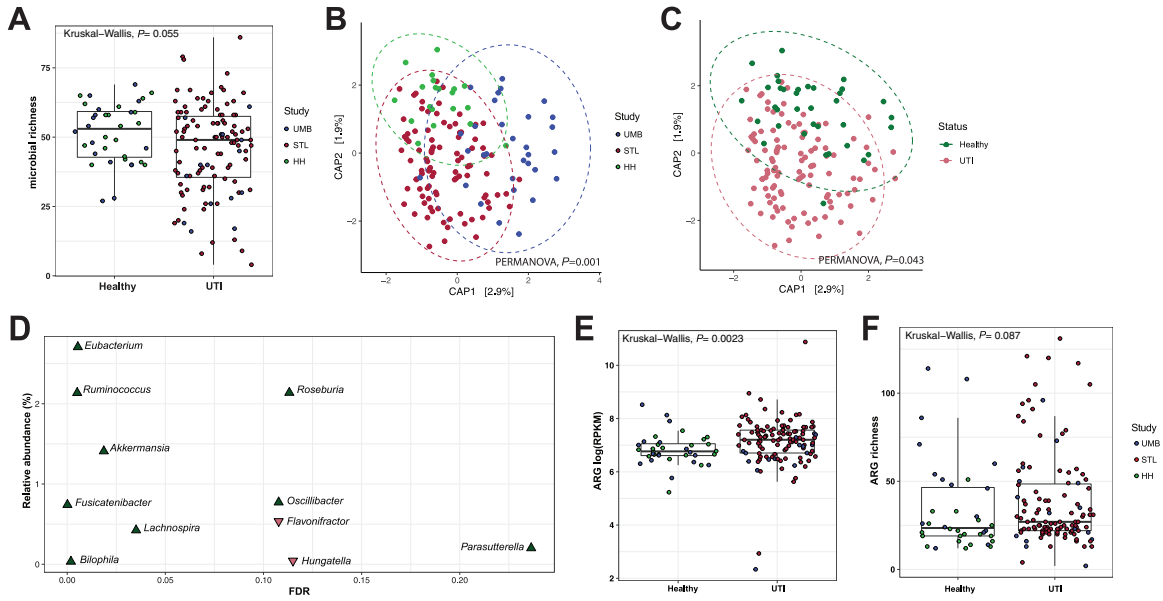

***Fig. 2:* Comparison of microbiomes between healthy and UTI individuals.** 20 published microbiomes from a healthy humans study (HH), and 31 published microbiomes from an rUTI study (UMB) were included for cross-cohort comparisons with our samples (STL). (A) Richness is higher in healthy microbiomes compared to UTI (Kruskal–Wallis, P = 0.055). Box indicates first and third quartiles, and whiskers extend to data within 1.5 times the interquartile range (IQR). Line in box indicates median. Datapoints beyond 1.5 times IQR are considered outliers. (B) Microbiomes were significantly different by study (PERMAOMVA, P = 0.001) but (C) Healthy and UTI microbiomes were significantly different even after accounting for study effect (PERMANOVA, P = 0.043). (D) Differentially abundant taxa at the genus level were identified using MaAsLin2. Green and upwards pointing triangles signify taxa enriched in healthy microbiomes, while red and downwards pointing arrows signify taxa enriched in UTI individuals. X-axis denotes the false discovery rate (FDR), and Y-axis shows relative abundance. (E) UTI microbiomes had higher numbers of antimicrobial resistance genes (ARGs) as identified by ShortBRED (Kruskal–Wallis, P = 0.0023). X-axis shows healthy or UTI groups, while Y-axis indicates the number of ARG hits as measured by Reads Per Kilobase of reference sequence per Million sample reads (RPKM). (F) Richness of ARGs was not significantly different between the two groups (Kruskal–Wallis, P = 0.087).

AST score (Kruskal–Wallis, $P < 0.001$, Fig. 3G). Corresponding urinary isolates from urinary tract colonizing lineages were not significantly elevated in AST score (Kruskal–Wallis, $P = 0.13$, Fig. 3H).

Discussion

We enrolled a prospective cohort of 125 patients with UTI to investigate the relationship between gut ARO colonization and rUTIs, controlling for clinical characteristics. In this select patient population, we did not identify specific, independent clinical characteristics associated with rUTI. We then utilized metagenomics to investigate the gut–bladder axis. Here we show that the gut microbiome in people with UTI is distinct from that of healthy individuals, reaffirming the role of gut microbiome dysbiosis in UTI.[9,13,15] In particular, the genera *Parasutterella*, *Akkermansia*, and *Bilophila* were depleted in intestinal samples of subjects with UTI in our cohort, consistent with previous findings.[13] However, when we compared UTI patients in our cohort with recurrence during the study period and those without, we found no significant gut microbiome differences. Instead, our findings point to asymptomatic colonization of the urinary tract as a significant distinguishing factor among gut microbiomes. Patients with urinary tract colonization displayed elevated gut *E. coli* abundance at post-antimicrobial, asymptomatic timepoints. This finding of *E. coli* blooms in the gut has been previously observed,[14] though importantly, the previous study utilized culture-based quantification while our metagenomic observations are limited in subspecies taxonomic resolution. Further subsetting the urinary colonized group into recurrence and non-recurrence samples found *B. xyalnisolvens* to be the singular taxon significantly elevated in the non-recurrent group, indicating the lack of broad taxonomic differences. Nevertheless, *Bacteroides* are commensals whose member species are under active investigation for probiotic development.[29] Their elevated presence may reflect a protective effect via competition in the gut microbiome,[30] despite urinary tract colonization by UPEC.

Urinary tract colonization was associated with elevated phenotypic resistance among gut isolates, but not urinary isolates. This finding underlines the gut microbiome's role in selection for specific resistance types during UTI, as reflected in elevated ARG

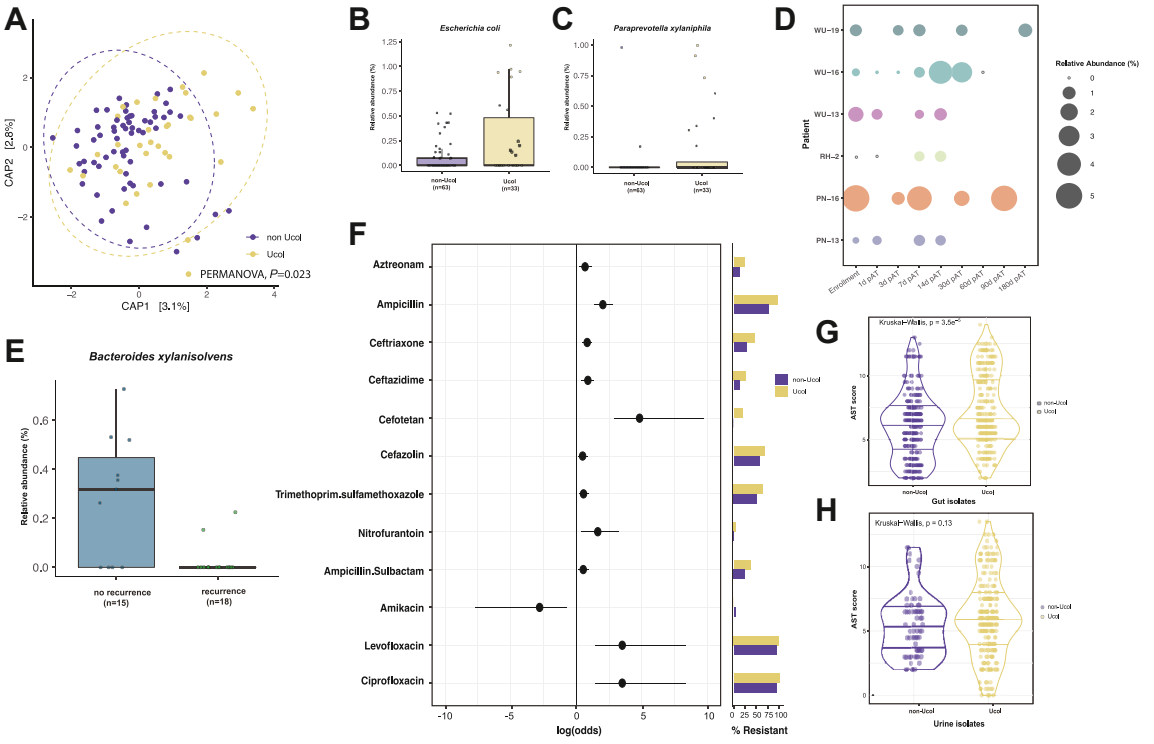

***Fig. 3:* Urinary tract colonization corresponds to significant differences in gut microbiome at days 7–14 post-abx.** (A) Taxonomic compositions of microbiome samples from days 7–14 post-abx were significantly different between urinary tract colonized (Ucol) and non-colonized patients (non-Ucol), even after accounting for age and treatment drug (PERMANOVA, $P < 0.05$, $n = 96$). (B and C) MaAsLin2 identified two taxa to be differentially abundant in Ucol patients: *Escherichia coli* and *Paraprevotella xylaniphila*. Box indicates first and third quartiles, and whiskers extend to data within 1.5 times the interquartile range (IQR). Line in box indicates median. Datapoints beyond 1.5 times IQR are considered outliers. (D) Ucol patients experience *E. coli* "blooms' in gut as measured by relative abundance. X-axis corresponds to sampling timepoint (S1: enrollment; S2: end of abx; S3: day3 post-abx; S4: day7; S5: day14; S6: day30; S7: day60; S8: day90; S11: day180). Y-axis rows and bubble colors correspond to patient ID, bubble size denotes relative abundance. Empty circles show 0.00% relative abundance in a sequenced sample. (E) *Bacteroides xylanisolvens* was the singular differentiating taxon between Ucol patients with recurrence, and Ucol patients without. (F) Firth's penalized likelihood logistic regression of AST results found gut isolates from Ucol lineages to be enriched in resistance for 11 of 23 tested drugs. Gut isolates from non-Ucol lineages were enriched in resistance to imipenem and meropenem. Circles indicate the odds ratio, while lines show the 95% confidence interval. Bars on the right show the percent of isolates from each group that are resistant to each drug. (G) Ucol gut isolates were significantly higher in AST score compared to non-Ucol gut isolates. Lines in violin plots show quartiles of distribution for each group. (H) Corresponding urinary isolates were not significantly different in AST score between Ucol and non-Ucol groups.

abundance, but not Shannon index, compared to healthy controls. A previous study of this cohort demonstrated the presence of 'hidden' ARGs among UPEC lineages which appeared after the diagnostic isolate, likely gained through mobile genetic elements enriched in the gut microbiome.[15] While urinary isolates belonging to the same lineage as the causative pathogen do not appear to maintain high resistance profiles during asymptomatic colonization,[31] it is plausible for a highly resistant gut isolate to migrate and cause recurrence in the urinary tract. Further research is needed to elucidate the migratory dynamics of UPEC in the host.

We acknowledge important limitations to this study. First, this study contains a select cohort of majority female patients with rUTIs caused by AROs, most of which were *E. coli*. Thus, the findings may not be generalizable to all populations of people with UTIs caused by diverse uropathogens. Another limitation is that the study was underpowered to detect risk factors for recurrence, which would require much larger sample sizes. Instead, we utilized the clinical characteristics to control for potential confounding factors prior to investigating the gut microbiome and urinary tract colonization. Finally, our gut microbiome comparisons are reliant on relative abundance predictions, significance testing, and sparse data—the latter of which can cause bias particularly in calculating odds ratios. Follow-up studies utilizing larger cohorts, diverse models, and strain-resolved metagenomic sequencing are warranted to generalize these findings. The strengths of our study include the well delineated, multicenter, prospective cohort design and our examination of the role of the

gut–bladder axis in rUTI while controlling for potentially-confounding, patient-level, clinical characteristics. Altogether our findings link *E. coli* populations in the gut microbiome to UPEC urinary tract colonization, providing further support for ongoing investigations of gut-targeting rUTI therapeutics.

Contributors

Conceptualization, J.H.K., E.R.D., C.-A.D.B., G.D., R.T., and J.C.; resources, J.H.K., E.R.D., G.D., K.A.R., S.S., C.C., M.H.B., and E.L.S.; investigation, R.T., T.H., A.T., M.A.W., B. Wang, Z.H.I., S.R.S., A.W.B., K.R.F., B.X., B. Williams, P.C.-T., E.L., and J.H.K.; data curation, K.A.R., K.B.N., M.A.O., J.C., and R.T.; bioinformatics and statistical analysis, K.A.R., K.B.N., M.A.O., R.T. and J.C.; writing—original draft, J.C., K.A.R., K.B.N., M.A.O., and R.T.; writing review & editing, J.C., R.T., K.B.N., T.H., K.A.R., M.A.W., M.A.O., V.J.F., A.W.B., B. Williams, P.C.-T., E.L., C.-A.D.B., E.R.D., J.H.K., and G.D.; visualization, R.T., J.C.; supervision, J.H.K., E.R.D., C.-A.D.B., and G.D.; project administration, K.A.R. and J.H.K.; funding acquisition, V.J.F., J.H.K., E.R.D., C.-A.D.B., and G.D.

Data sharing statement

Sequence data generated from this study are available on NCBI SRA under PRJNA682246. Metagenomic analysis scripts and relevant metadata are available on Github: https://github.com/jhchoi17/rUTI_gutmicrobiome.

Declaration of interests

E.R.D. reports grants from Theriva Biologics, trial enrollment support and consulting fees from Ferring, and consulting fees from Seres. V.F. reports grants from The Foundation for Barnes-Jewish Hospital, grants from Doris Duke Charitable Foundation, grants from NIH/NCATS (project numbers KL2TR002346, UL1TR002345), and royalties/licenses from Elsevier (Goldman-Cecil Medicine, 2-Volume Set, 27e). She has served various roles at the Infectious Diseases Society of America (Board of Directors 2017–2020, IDSA Leadership Institute 2018–2022, Editor-in-Chief Search Committee Chair of the Open Forum Infectious Disease Journal 2022). Her spouse is a consultant and former Senior Vice President/Chief Medical Officer at Cigna/Express-Scripts. M.A.O. reports consulting fees from Pfizer. C.A.B. reports paid roles as editor at the Journal of Clinical Microbiology, and unpaid roles with the Clinical and Laboratory Standards Institute. She has served as Chief Clinical Officer at Pattern Bioscience since 2022 and holds shares. All other authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.eclinm.2024.102490.

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