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ORIGINAL RESEARCH

Differential Effects of Uropathogenic and Non-Uropathogenic *E. coli* on the Mouse Urobiome and Urine NGAL Levels

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Objective: To determine whether urine neutrophil gelatinase-associated lipocalin (uNGAL) or urobiome alterations can differentiate urinary tract infections (UTI) from asymptomatic bacteriuria (ASB).

Methods: Female 8-week-old C57BL/6 mice were instilled with either *Escherichia coli* CFT073 (UTI model, n=12), *E. coli* 83972 (ASB model, n=12), or saline (control, n=3). uNGAL was measured daily for 3 days post-instillation. Urobiome composition was assessed pre- and post-instillation using 16S rRNA sequencing. At day 3, kidneys were harvested for culture. Comparisons were made across groups for uNGAL levels and urobiome diversity.

Results: Baseline β diversity did not differ between groups. Post-instillation, β diversity significantly differed across groups (p=0.01), driven by increased relative abundance of *E. coli* in UTI mice compared to ASB mice. Median uNGAL levels increased significantly in both UTI and ASB groups relative to controls, but no significant difference was observed between UTI and ASB groups.

Conclusion: Introduction of a uropathogenic *E. coli* strain reduced urobiome diversity, while a non-uropathogenic strain did not, suggesting strain-specific effects on microbial ecology. Bladder instillation itself also altered the urobiome. Elevated uNGAL levels were observed in both UTI and ASB models, indicating that while uNGAL reflects bacterial exposure, it does not distinguish between uropathogenic and non-uropathogenic *E. coli*. These findings highlight urobiome analysis as a potential tool for differentiating UTI from ASB, whereas uNGAL alone is insufficient.

Keywords: urinary tract infection, biomarker, mice, microbiome, asymptomatic bacteriuria

Introduction

Urinary tract infections (UTI) are one of the most common bacterial infections across all age groups. The diagnosis of UTI consists of the combination of inflammation, frequently measured by pyuria and a positive urine culture in a symptomatic person. In people without chronic medical conditions, the diagnosis of UTI is often made clinically and can be augmented by use of the urine dipstick. However, accurately diagnosing UTI in people with neurogenic lower urinary tract dysfunction (NLUTD), is challenging as there is a lack of widely accepted standardized diagnostic guidelines. Further, the guidelines that do exist vary in terms of symptoms, degree of inflammation, and bacterial load considered to be indicative of a UTI.[1–4] The high rate of bacteriuria and frequency of urinary symptoms experienced by people with NLUTD underscores this challenge, as it is difficult to distinguish when a positive urine culture represents a UTI as opposed to asymptomatic bacteriuria (ASB).[5] Pyuria is often used clinically to distinguish UTI from ASB; however, it is sensitive, but not specific, for UTI in people with NLUTD.[6] Thus, a more accurate method to diagnose UTIs in people with NLUTD is needed.

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Studying biomarkers that are specific to the host–pathogen relationship is a promising way to identify novel methods to improve the accuracy of UTI diagnosis. One way to examine the host response is by measuring urinary antimicrobial peptides, a part of the innate immune response within the bladder.[7] One such antimicrobial peptide is urine neutrophil gelatinase associated lipocalin (uNGAL), which is produced and secreted in the urinary tract in response to infection.[8] The pathogenic side of the host–pathogen relationship can be assessed by analyzing the urobiome using sequencing-based methods that more accurately identify bacteria within the urobiome than the standard urine culture typically used within clinical labs.[9] Thus, comparing uNGAL and the urobiome in ASB and UTI mouse models may yield data regarding their utility in distinguishing these two clinical states.

While clinical research has reported that uNGAL has utility as a diagnostic biomarker for UTI in children and adults both with[10–12] and without NLUTD,[13] these studies are all limited by the lack of a gold standard method to differentiate ASB from UTI. The use of mouse models of UTI enables an objective assessment of how uNGAL and the urobiome respond to instillation of *E. coli*: a uropathogenic strain to model UTI versus a non-uropathogenic strain to model ASB. Therefore, the overall objective of this study was to determine if either uNGAL or changes in the urobiome can be used to differentiate UTI from ASB.

Materials and Methods

Mice

We used eight-week-old female C57BL/6 mice (stock no. 000664, The Jackson Laboratory, Bar Harbor, ME). Animals were allotted a 7-day acclimation period after arrival to the animal facility prior to initiation of this study. All animal work was approved by the Institutional Animal Care and Use Committee at the Biomedical Research Institute (Rockville, MD) under protocol number 20–01 and performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals to ensure animal welfare. We chose not to use a mouse model of NLUTD in this study as we first wanted to understand how uNGAL and the urobiome responded to both uropathogenic and nonuropathogenic strains of *E. coli* in an uninjured model before translating these results into a mouse model of NLUTD.

Bacterial Growth and Instillation

All work with bacteria was performed under sterile conditions in a biosafety cabinet. Bacteria were cultured as described in Hung et al.[14] *E. coli* CFT073 (uropathogenic strain to model UTI) and *E. coli* 83972 (non-uropathogenic strain to model ASB) were obtained from BEI Resources (Manassas, VA).

We used the methods described by Hung et al to induce UTI in the mice.[14] Mice were anesthetized using 2% laminar flow of isoflurane and placed in the supine position. Any urine in the bladder was expressed by gently pressing on the lower abdomen. A 24 g × ¾ inch sterile angiocath (Clint Pharmaceuticals, Old Hickory, TN) was attached to the prepared 1 mL syringe (Fisher Scientific, Waltham, MA) containing the inoculant. The angiocath was lubricated (DynaLub Sterile Lubricating Jelly, Amazon, Seattle, WA) and inserted transurethrally into the bladder. To insert the catheter, the instiller used one hand to spread and stabilize the periurethral tissue, while using their dominant hand to insert the catheter into the urethral meatus parallel to the table. The catheter was slowly advanced until resistance was felt. If resistance was appreciated, the catheter was withdrawn by several millimeters, the angle changed slightly, and readvanced until the bladder was reached, which was appreciated by insertion of most of the length of the angiocath into the urethra.[15] One hundred milliliters of 10^8 colony-forming units of the respective inoculant was slowly instilled into the bladder over a 1-minute period using a syringe pump (Harvard Apparatus, Hollison, MA) and allowed to dwell for one minute. Twelve mice were inoculated with the uropathogenic *E. coli* to model UTI, 12 mice were inoculated with the non-uropathogenic *E. coli* to model ASB, and 3 mice were inoculated with sterile phosphate-buffered saline (PBS) (control group).

Urine Collection

Mice were placed above sterile parafilm (Heathrow Scientific, Vernon Hills, IL) and the abdomen or lower back tapped to express urine.[16] Urine was aspirated from the sterile parafilm. Urine samples were collected before instillation of bacteria

or vehicle (pre-instillation) and 72 hours post-instillation (post-instillation). The urine was flash frozen immediately upon collection and stored at −80°Celsius.

Kidney Collection

After the 72-hour urine sample was collected, mice were humanely euthanized with an intraperitoneal dose of a combination of phenobarbital and phenytoin (Virbac, West Lake, TX) followed by cervical dislocation to ensure death. We confirmed death by monitoring for the absence of pulse, absence of respirations and absence of response to toe pinch. The procedure was carried out in accordance with the American Veterinary Medical Association's guidelines for the euthanasia of animals and was designed to minimize pain and distress. Kidneys were harvested using sterile technique at 72 hours post-instillation. After harvest, one kidney was harvested from each mouse, dipped in 70% ethanol, homogenized and plated on sterile TSB plates (Thermo Scientific, Waltham, MA) to assess for *E. coli* growth.

NGAL Measurement

uNGAL was measured using Mouse NGAL ELISA Kit (KIT 042, BioPorto, Denmark), according to kit instructions. Out-of-range samples were diluted 1:4 and re-analyzed, according to kit instructions. Urine creatinine was measured using Colorimetric Creatinine Assay Kit (ab204537, Abcam, Branford, CT), according to kit instructions.

DNA Isolation, Polymerase Chain Reaction (PCR) Amplification, and 16S rRNA Gene Sequencing

DNA was isolated using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD), according to kit instructions, with the exception that DNA was eluted from the column with nuclease-free water (Sigma-Aldrich, St. Louis, MO). Library preparation and sequencing were performed according to Illumina 16S Metagenomics Sequencing Library Sample Preparation Guide. Genomic DNA isolated from mouse urine samples was used to amplify the 16S rRNA gene variable regions V3-V4. The primers used for the amplification were 16S Amplicon PCR Forward Primer (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 16S Amplicon PCR Reverse Primer (5'TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVG-GGTATCTAATCC). The IDT-Illumina DNA-RNA UD Indexes Set A Tagmentation index kit (Illumina, San Diego, CA) was used for the index PCR. The amplicon was pooled and sequenced with MiSeq v3 600 cycle reagent kit (Illumina, San Diego, CA) with 15% PhiX spike-in (Illumina, San Diego, CA).

Taxonomic Identification

High-quality trimmed reads were obtained after low-quality reads were removed using Cutadapt.[17] DADA2 software was used to process reads, including filtering, dereplication, chimera removal steps, and construction of an amplicon sequence variant (ASV) abundance table.[18] The ASVs were annotated, using the Bayesian LCA-based taxonomic classification method,[19] which uses the National Center for Biotechnology Information (NCBI) database to achieve taxon identification. ASVs with taxa confidence scores less than 70% were assigned "unclassified/unknown". Low abundance ASVs were removed.

Statistical Analysis

The α (within-sample) and β (among-samples) diversity indices were computed, using statistical packages phyloseq,[20] vegan,[21] and philentropy[22] in R (R Foundation for Statistical Computing, Vienna, Austria). The α diversity measures were calculated for each sample at both timepoints. Richness (Observed, Chao1, and ACE) is the number of unique taxa per sample; more diverse samples have larger values. Evenness refers to the distribution of taxa per sample. More evenly distributed samples have larger values. Shannon and Simpson values include richness, evenness, and abundance in their calculations. The β diversity was used to determine whether instillation and/or time of analysis had an effect on the composition of the microbial communities.

Instillation groups and timepoints were compared using Wilcoxon rank sum tests for paired as well as unpaired α diversity indices. The PERMANOVA test of β diversity was performed using the vegan package in R (R Foundation for Statistical Computing). uNGAL values were compared between groups using Kruskal Wallis with post-hoc Dunn or Mann–Whitney U as appropriate.

Sample Size Justification

We powered our study based on the hypothesis that mice instilled with a uropathogenic strain of *E. coli* would have uNGAL levels consistent with prior data in the literature,[8] and that mice instilled with a non-uropathogenic strain would have uNGAL levels consistent with those reported for people with ASB.[12] Thus, we used these data to complete our a priori sample size calculation to determine the minimal number of mice that needed to be used to detect a meaningful difference in uNGAL values between our model of UTI and ASB. The control mice were included to ensure we had appropriate negative controls but were not part of our sample size calculation in order to minimize the number of mice used in this work.

Results

Mouse Urine Microbiome

We used 16S rRNA gene sequencing to assess the urobiomes of 27 female mice before and after instillation of PBS (control, n=3), a non-uropathogenic strain (ASB, n=12), or a uropathogenic strain (UTI, n=12) of *E. coli* (Figure 1). β diversity analysis revealed no significant difference in the microbiomes of the 3 sets of pre-instilled mice (Figure 2A, PERMANOVA p=0.77). This lack of significant differences was confirmed by α diversity metrics (Supplementary Figure 1). However, in the post-instilled mice, β diversity analysis did detect significant differences of the microbiome amongst the three groups (Figure 2B, PERMANOVA p=0.01). This significant difference was confirmed by α diversity metrics (Supplementary Figure 2). The differences seen in the post-instilled mouse urine was not due to differences between the pre- and post-instilled control mice as determined by β diversity (Figure 2C, PERMANOVA p=0.16) or α diversity (Supplementary Figure 3). Although β diversity analysis (Figure 2D, PERMANOVA p = 0.27) and some α diversity metrics (Supplementary Figure 4A-C and E) detected no significant difference between the pre- and post-instilled ASB mice, some α diversity metrics (Evenness & Simpson) detected some differences (Supplementary Figure 4D–F, Wilcoxon Rank Test both p<0.05). These differences in Evenness and Simpson may be due to the increased relative abundance of the genera *Escherichia* (yellow) and/or *Staphylococcus* (blue) in some ASB mice after instillation (Figure 1). In contrast, the microbiomes of pre- and post-instilled UTI mice differed significantly as determined by both β diversity (Figure 2E, PERMANOVA p = 0.01) and all α diversity metrics (Supplementary

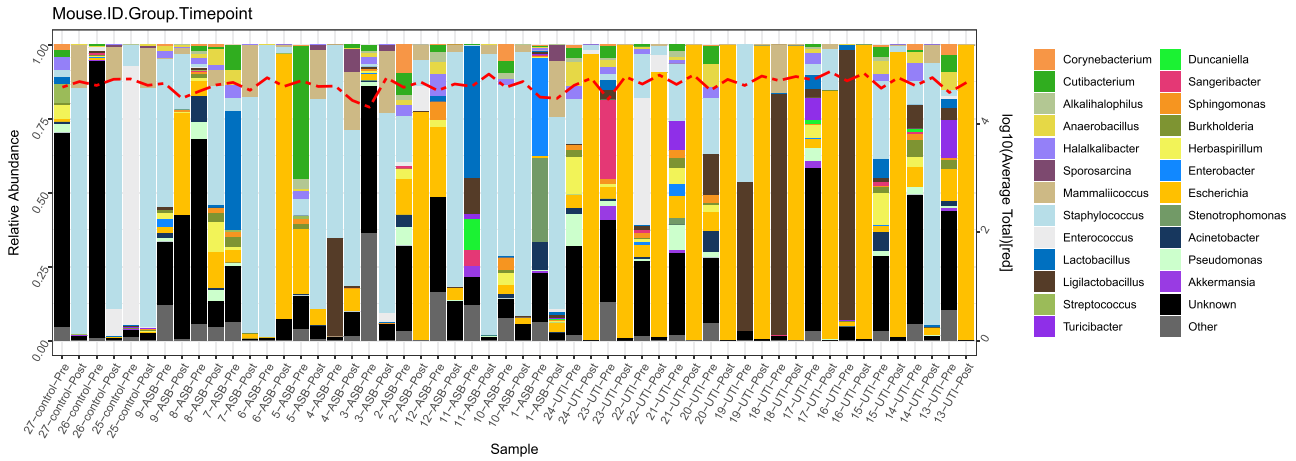

Figure 1 Relative abundance of V3-V4 16S rRNA gene sequences obtained by expressed urine of individual mice pre- and post-instillation of PBS (control), non-UPEC (ASB), or UPEC (UTI). Relative abundance of the top 25 bacteria at genus level. Lower abundant taxa were aggregated into taxa group "Other". Unknown represents unidentified sequences. Red dashed line represents the total reads of sequences in each sample sequence.

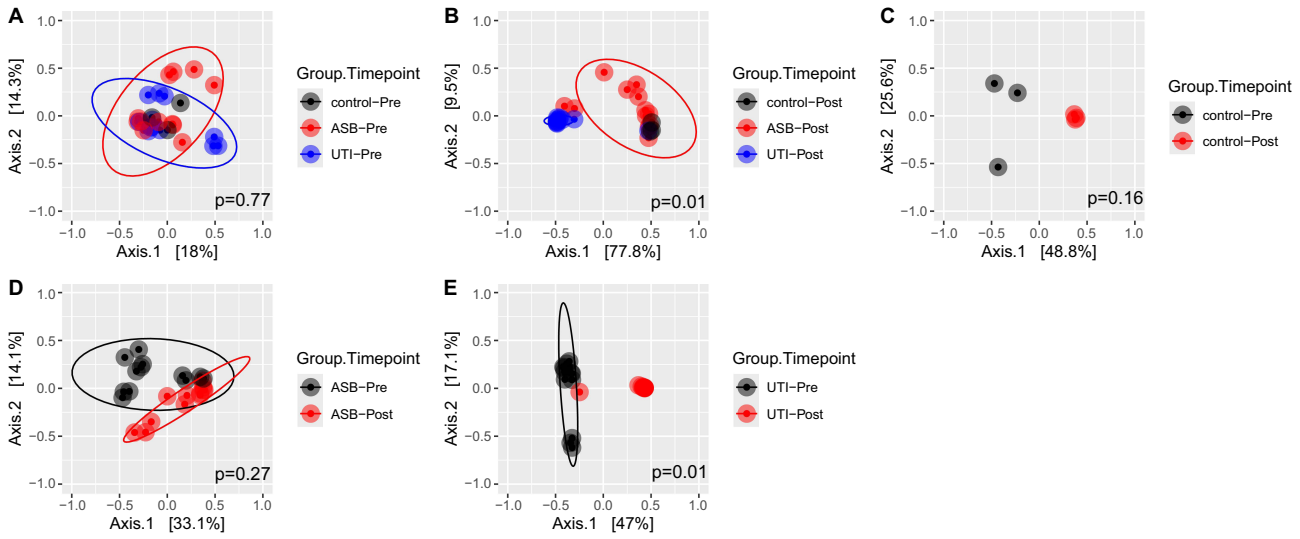

Figure 2 Beta diversity analyses of V3-V4 16S rRNA gene sequences obtained by expressed urine of individual mice pre- and post-instillation of PBS (control), non-UPEC (ASB), or UPEC (UTI). PCoA analysis using Bray Curtis dissimilarity matrix of (**A**) the pre-instillation groups only, (**B**) the post-instillation groups only, (**C**) pre- and post-instillation of the control group only, (**D**) pre- and post-instillation of the ASB group only, and (**E**) pre- and post-instillation of the UTI group only. P-values were calculated using PERMANOVA statistic.

Figure 5). This difference was driven by the increased relative abundance of the genus *Escherichia*, which was substantially greater in the post-instilled UTI mice compared to the post-instilled ASB mice (Figure 1).

uNGAL Levels by Group and Over Time

Median post-instillation uNGAL levels were significantly higher in both the post-instilled UTI and ASB mice compared to the control group. There was no significant difference in the median post-instillation uNGAL levels between mice in the UTI and ASB groups (Figure 3, left). Median uNGAL levels were significantly higher in mice inoculated with ASB (1263 pg/mL) and UTI (1494 pg/mL) compared to the PBS (73 pg/mL). However, median uNGAL levels did not differ significantly between mice instilled with the uropathogenic or non-uropathogenic *E. coli*. The median change in uNGAL (ΔuNGAL) levels (defined as uNGAL level post-instillation minus baseline levels) were significantly higher in both groups given the ASB strain (1193 pg/mL) and UTI strain (1456 pg/mL) compared to the PBS (22.88 pg/mL). However,

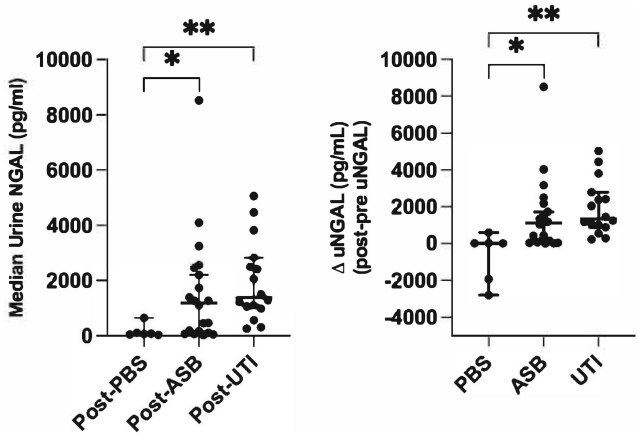

Figure 3 Mice inoculated with either non-uropathogenic *E. coli* strain 83972 or uropathogenic *E. coli* strain CFT073 had significantly higher uNGAL levels than mice inoculated with PBS. Mice were inoculated with either PBS, *E. coli* 83972 (eg, ASB in figure), or *E. coli* CFT07 (eg, UTI in figure) 3 and uNGAL was measured by ELISA after 72 hours. *Left:* delta NGAL in mice instilled with either ASB, UPEC, or PBS. *Right:* median uNGAL levels in post-instillation urines. Bars represent the median and 95% confidence interval. Note that there is one outlier in each of the 83972 and CFT073 receiving groups that is not show here. Statistics: Kruskal–Wallis test where * indicates p≤0.05 and ** indicates p≤ 0.01.

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median ΔuNGAL levels did not differ significantly between the ASB and UTI groups. Similar results were observed for the change in uNGAL from pre- to post-installation (Figure 3, right). Normalizing uNGAL levels with urine creatinine produced the same results (data not shown).

Three mice had significant bacterial growth in kidney culture. There was no association between either Shannon diversity or NGAL levels of these three mice. Neither Shannon Diversity Index nor NGAL could identify the mice whose kidneys had high bacterial burdens.

Discussion

Here, we report changes in the urobiome of mice following instillation of either a UTI or ASB strain of *E. coli*. Although we found no difference in the pre-instillation urobiomes amongst the three groups, we observed differences in the post-instillation urobiomes between mice instilled with the ASB and UTI strains; these differences were driven by increased abundance of *E. coli* in those instilled with the UTI, as opposed to the ASB, strain. We also show that uNGAL is higher in mice following instillation with both the UTI and ASB strains of *E. coli* compared to the pre-instillation values. This work is novel as it examines how the urobiome and a specific antimicrobial peptide (ie, uNGAL) respond to strains with differing degrees of uropathogenic potential, which is a gap within the clinical literature.

We chose to study uNGAL over other cytokines as there is more robust literature suggesting that uNGAL levels are higher in people with UTI compared to those with ASB, and that uNGAL is a specific marker of UTIs in children with NLUTD and positive urine cultures.[11,12] Similar data are limited for other cytokines. Our results in mice suggests that the uNGAL response alone is not reflective of the pathogenicity of the instilled bacteria, but rather simply a response to being exposed to the Gram-negative bacterium *E. coli*. One plausible explanation could be that NGAL secretion in the urinary tract occurs in response to lipopolysaccharide; thus, Gram-negative bacteria expressing LPS should induce uNGAL secretion.[8] However, low levels of uNGAL reported in some people with positive standard urine cultures suggests that this is not the case.[11,12] Another possibility is that the pathophysiology of UTI in people may be more complex than modeled in mice. A third possibility involves the choice of *E. coli* strains. While the two strains used in this work were initially isolated from a person with a clinically diagnosed UTI (*E. coli* CFT073) and a person without symptoms (*E. coli* 87972), it is possible that the latter has uropathogenic potential and thus can cause infection under certain conditions, as has been reported in the literature.[23]

The difference in the post-instillation urobiomes is a notable finding of this work. The fact that *Escherichia* predominates the post-instillation urobiome of the uropathogenic group is not surprising: It is consistent with the hypothesis of clonal expansion in the setting of UTI. Indeed, this has been reported in children with UTI.[24] However, the *Staphylococcus* predominance in the non-uropathogenic group is unexpected. While contamination of collected urine is a possible cause, *Staphylococcus* was not the predominant microbe in the pre-instillation urobiome, which was collected in the same manner as the post-instillation urobiomes, suggesting that contamination may not be the main cause of this finding. However, it is notable that the post-instillation urobiomes in the control group were also predominated by *Staphylococcus*, suggesting either contamination of the saline, which while possible is unlikely given the sterile technique used, or that the act of flushing the bladder itself alters the urobiome.

We chose to avoid a mouse model of NLUTD for several reasons, the primary being that describing the relationship between uNGAL, the urobiome, and the instilled microbe in a normal mouse is a necessary first step before translating these results into a model of NLUTD. However, another consideration is that most mouse models of NLUTD require antibiotics following the spinal injury procedure, which will alter the baseline urobiome. As there is a lack of longitudinal data suggesting how long it takes the urobiome to reach steady state following spinal cord injury, the decision about how long to survive the mice following spinal cord injury would not be data driven. One study of the infant gut microbiome reported normalization occurs over 12 months after antibiotics,[25] suggesting that reaching a steady state can require a significant time. As we are interested in the change in the urobiome, it was important to have a non-dysbiotic pre-instillation urobiome. Therefore, we decided to first evaluate these relationships in wild-type mice, and then translate these findings into mice with spinal cord injury.

This work had several challenges and limitations. Our main challenge involved sequencing the mouse urobiome given the low volume of mouse urine and the low microbial biomass in that urine. Further, obtaining urine from mice is

challenging, and there is likely a degree of contamination from the perineum despite all efforts to obtain a clean catch. However, all urine for this work was collected in this manner, thus allowing comparisons between groups. Other limitations include the knowledge that uNGAL is a biomarker for acute kidney injury (AKI) and, therefore, high uNGAL levels of mice could suggest concomitant AKI in the setting of UTI.[26] However, AKI in the setting of UTI would most likely be due to severe infection, which is unexpected in the non-uropathogenic group. The mouse model of UTI has its own limitations, such as mice spontaneously clearing an infection and the mouse not fully replicating the pathophysiology of a human UTI.[27] Finally, several host factors could affect our results. While we could control for several of these, such as age and sex, we could not control for all host factors, which is a limitation of our work.

Conclusion

Here, we show the diversity of the urobiome decreases when a uropathogenic, but not a non-uropathogenic, strain is introduced into the bladder. Further, we suggest that the act of flushing the bladder itself alters the urobiome. Finally, we show that uNGAL levels increase in mice instilled with either a uropathogenic or a non-uropathogenic strain of *E. coli*, suggesting that uNGAL alone cannot identify uropathogenic potential of bacteria and perhaps non-uropathogenic *E. coli* may still illicit an inflammatory response.

Animal Studies

Institutional Animal Care and Use Committee Number: Biomedical Research Institute 20-01. University of Pittsburgh 23124147.

Disclosure

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