SUPPLEMENTAL METHODS

Study Cohorts: Genetic samples were obtained from three clinical cohorts with and without a history of UTI or VUR as described below. All genomic DNA samples were obtained from immortalized lymphoblastic cell lines.

(i) The Randomized Intervention for Children with Vescoureteral Reflux (RIVUR) Trial (ClinicalTrial.gov Identifier NCT00405704) enrolled 607 children aged 2–71 months with grade I-IV VUR diagnosed by radiographic voiding cystourethrogram after a first or second febrile or symptomatic UTI (1). UTI was defined as a culture proven infection with a single organism ≥ 50,000 CFU/mL on a catheter specimen or ≥ 100,000 CFU/mL on a clean catch specimen with evidence of pyuria on urine analysis. We utilized DNA samples obtained from 424 females enrolled in the RIVUR study.

(ii) The Careful Urinary Tract Infection Evaluation (CUTIE) Study observed 195 children aged 2–71 months who had one to two febrile or symptomatic UTIs but did not have diagnosed VUR (2). We utilized DNA samples obtained from 160 females enrolled in the CUTIE Study.

(iii) For case-control comparison, we utilized 482 DNA samples obtained from the National Institute of Neurological Disorders Repository supplied by the Coriell Institute. Our control cohort consisted of non-Hispanic Caucasian females with no history of VUR and UTI.

Human Genotyping: All samples were genotyped in replicates by Transnetyx Automated Genotyping Services (Cordova, TN, UA) utilizing ThermoFisher (Waltham, MA) Scientific TaqMan® SNP Genotyping Assay C_2434509_10 and standard Applied Biosystems (ABI, Foster
City, CA) TaqMan® assay conditions: denaturing for 95°C for 10 minutes followed by 40 cycles of denaturing at 95°C for 15 seconds and 60°C for 1 minute combined annealing and extension. TaqMan® Genotyping Master Mix and TaqMan® GTXpress™ Master Mix were used and diluted to 2X with the TaqMan® SNP assay probe diluted to 4X. Genomic samples were diluted to 1-10 ng of DNA per manufacturer’s instructions.

Any ambiguous genotyping results were subsequently PCR amplified and sequenced for manual verification. A random selection of samples with known rs1263872 genotype from each TaqMan® SNP PCR assay were also sequenced for internal validity. Samples were amplified using Phusion™ High-Fidelity DNA polymerase, IDT oligo PCR primers, double-distilled PCR-grade water, and DNA template in concentrations and conditions according to manufacturer’s instructions. PCR products were sequenced by the University of Tennessee core facility using an ABI 3130XL Genetic Analyzer and SNP genotypes were verified with Technelysium Chromas DNA Sequencing Software (South Brisbane, Australia). The following primers were used for PCR amplification and sequencing: forward sequence: 5’- GAT CTG ACA GCC TAG GAG TGC -3’ and reverse sequence: 5’- GGG GTC GCT TTG CGC TTG -3’.

**E. coli Strains:** E. coli strain CFT073 was isolated from the blood of a patient with acute pyelonephritis while UPEC strains J96, 536 (kindly provided by Dr. Harry Mobley, University of Michigan, United States), and UTI89 were isolated from human urine as previously published (3-6). UTI89Δ*fimH* (provided by Dr. Matt Mulvey, University of Utah, United States) does not produce adhesive type 1 pili (7). Additional UPEC strains were obtained from girls evaluated at Nationwide Children’s. The diagnosis of UTI was made by a positive urine culture and the presence of pyuria according to American Academy of Pediatrics Guidelines (8). UPEC strain
MDR58 was obtained from a 7 year old girl presenting with fever and urinary symptoms. UPEC strain MDR12 was isolated from the urine of a 2 year old girl and MDR51 was from a 7 year old girl. These children presented with lower urinary tract symptoms, dysuria, and were afebrile. All children had normal urinary tract anatomy and no documented history of dilating VUR. The antibiotic susceptibility testing for UPEC strains MDR51 and MDR58 is previously published (9). UPEC strain MDR12 showed antibiotic resistance to cefazolin, cefpodoxime, ceftazidime, ceftriaxone, ciprofloxacin, levofloxacin, and trimethoprim-sulfamethoxazole. It also showed intermediate resistance to amoxicillin/clavulanate, cefepime, and nitrofurantoin. The laboratory adapted fecal/commensal isolate MG1655 was used as a control (10).

**Multiplex PCR of UPEC serotypes and virulence genes:** Genomic DNA was extracted from UPEC cultures grown statically for 24 hours using the phenol/chloroform method. Multiplex PCR was performed with the QIAGEN Multiplex PCR Kit using 100ng gDNA and pooled sets of primers to characterize O-antigen serogroups, virulence factors, and phylogenetic grouping of each strain as described previously (11-13). Amplification products were visualized on a 2% agarose gel.

**UPEC Hemagglutination:** UPEC hemagglutination was determined in guinea pig erythrocytes as previously described (14). Briefly, UPEC cultures were statically grown in LB broth for 24 hours and standardized to $A_{600} = 0.8$ ($\sim 10^8$ CFU/mL). Bacteria were then pelleted, resuspended in 0.1 mL PBS/mL of culture, and serially diluted twofold in PBS. 25 $\mu$L of bacteria dilutions were incubated for 30 minutes with an equal volume of 3% (vol/vol in PBS) purified erythrocytes. Numbers reported are the lowest dilution of bacteria to cause hemagglutination. Hemagglutination
caused by mannose-sensitive type 1 fimbriae was determined by the inhibition of hemagglutination in the presence of 50 mM d-mannose with undiluted UPEC.

**Expression of recombinant RNase 7 peptides:** Using PCR, the *RNASE7* peptide coding *RNASE7*\textsubscript{103Ala} sequence was amplified from human kidney cDNA and cloned into the *E. coli* expression vector pDEST17 (Invitrogen, Carlsbad, CA, USA) as previously described (15-17). Forward and reverse sequencing confirmed the absence of PCR errors (Eurofins, Luxembourg). Next, the *RNASE7*\textsubscript{103Pro} variant was generated using the Quick-change Multi Site-Directed Mutagenesis kit (Agilent, Santa Clara, CA) per the manufacturer’s instructions. Forward and reverse sequencing confirmed the absence of PCR errors (Eurofins). Full-length *RNASE7*\textsubscript{103Ala} and *RNASE7*\textsubscript{103Pro} containing plasmids were transformed into *E. coli* BL21 AI cells (Invitrogen) to allow for L-arabinose inducible expression. Recombinant RNase 7 proteins were purified, refolded, and dialyzed as previously described (15, 17). Protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). RNase 7 peptide was visualized by SDS-polyacrylamide gel electrophoresis, followed by Coomassie staining.

**Recombinant peptide antibacterial assay:** The antimicrobial activity of recombinant RNase 7 was evaluated against UPEC using colony count reduction assays as previously described (15). UPEC cultures were statically grown in LB broth for 24 hrs. \(10^5\) colony forming units (CFU)/mL of UPEC pyelonephritis and cystitis strains were incubated with 2.5 μM *RNASE7*\textsubscript{103Ala} or *RNASE7*\textsubscript{103Pro} for 90 minutes at 37°C (18-20). The antimicrobial activity of RNase 7 was analyzed by plating the incubation mixtures and determining the CFUs the following day. Repeat testing was performed on all bacterial isolates in duplicate.
**Affinity Binding Assay for LPS:** LPS binding was assessed using the fluorescent probe Bodipy TR cadaverine as previously described (21-24). The displacement assay was performed by adding equal concentrations of the RNase 7 peptides (2.5 μM) or a negative control peptide (RNase A) to LPS (1 μg/mL) labeled with BC in 5 mM Hepes buffer at pH 7.5. Fluorescence measurements were performed in the Spectramax M2 multimode reader (Molecular Devices, Sunnyvale, CA, USA). The BC excitation wavelength and emission wavelength were 584 nm and 620 nm, respectively. Displacement was quantified using the equation \( OF = (F_0-F)/(F_0-F_{max}) \). \( OF \) is the occupancy factor, \( F_0 \) the fluorescence intensity of BC alone, \( F_{max} \) the BC intensity in the presence of LPS at saturation, and \( F \) is the intensity of the LPS/BC mixture (24).

**Generation of stable RNase 7 retroviral constructs and urothelial cell transduction:** The PINCO retroviral transfer plasmid was kindly provided by Dr. Jianhua Yu (The Ohio State University, Columbus, Ohio, United States) (25). The PINCO retroviral vector permits \( RNASE7 \) expression from the 5’ long terminal repeat as well as the enhanced green fluorescent protein (EGFP) from an internal cytomegalovirus immediate early promoter as described (9, 26, 27). cDNA encoding the \( RNASE7^{103\text{Ala}} \) or \( RNASE7^{103\text{Pro}} \) open reading frame was cloned into the \( \text{BAMHI} \) and \( \text{EcoRI} \) sites of PINCO. Following confirmation of cloning by bidirectional DNA sequencing (Eurofins), each construct was prepared for virus production by endotoxin-free maxiprep (Qiagen, Carlsbad, CA). \( RNASE7^{103\text{Ala}} \) or \( RNASE7^{103\text{Pro}} \) overexpressing PINCO retroviral supernatants and PINCO control supernatants were generated by transient transfection of the Phoenix-Ampho packaging cell line with plasmid DNA expressing the VSV-G protein as previously described (9, 26).
Human UROtsa cells, which were isolated from primary culture of human urothelium and are previously characterized, were transduced with the resulting *RNASE7* overexpression constructs and control PINCO constructs as described (9, 26, 28, 29). Due to low initial infection efficiency, EGFP+ cells were isolated by fluorescent activated cells sorting (FACS) using a BD Influx™ cell sorter (DB Biosciences, San Jose, CA) to establish a polyclonal population with >90-95% EGFP+ expression (9).

**SDS-PAGE Western blot and ELISA:** Protein extraction and Western blot on cell culture lysates were performed as previously described (17, 30). Primary antibodies included: monoclonal mouse anti-RNase 7 (clone CL0223, Sigma-Aldrich, St. Louis, MO, USA) and rabbit polyclonal anti-GAPDH (Cell Signaling, Danvers, MA, USA). The RNase 7 ELISA was performed as previously published (31).

**Extracellular antimicrobial assays:** Three-day old media from control and *RNASE7* expressing UROtsa cells was collected an inoculated with 2x10^5 CFU/mL UPEC for 90 minutes at 37°C. After this incubation, UPEC containing media were serially diluted and plated onto LB-agar plates. Following an overnight incubation at 37°C, the number of surviving UPEC were enumerated.

**UPEC attachment and invasion assays:** Bacterial attachment and invasion assays were performed as described on confluent UROtsa cells cultured in 24 well plates (9). After replacing fresh medium one hour before UPEC challenge (to minimize extracellular UPEC killing), host cells were infected with 2x10^5 CFU/well UPEC for two hours at 37°C. UPEC contact with host cells was expedited by centrifugation of plates at 1000 rpm for five minutes. Cell integrity was
assessed by visual inspection and trypan blue exclusion assays. After UPEC incubation, one set of infected cells (4 wells) were lysed in 0.1% Triton X-100 in phosphate buffered saline (PBS) and plated onto LB-agar plates to enumerate the total number of UPEC per well (extracellular and intracellular) to account for bacterial growth over the time period of the experiment. To assess attachment, another set of infected cells (4 wells) were washed with PBS and then lysed in 0.1% Triton X-100 and plated on LB-agar plates. The percentage of adherent bacteria was determined as the number of CFU recovered after PBS washing divided by the total number of CFU in each well.

UPEC cellular invasion was determined using a gentamicin protection assay (32, 33). An additional set of infected cells (4 wells) were washed with PBS and incubated for an additional two hours in gentamicin containing media (100 µg/mL) to kill any extracellular bacteria, which was confirmed by plating media samples. After PBS washing, cells were lysed and plated onto LB-agar plates. The percentage of intracellular bacteria was determined as the number of CFU recovered after cell lysis following incubation in the presence of gentamicin divided by the total number of CFU.

**Statistical analysis:** Genetic data are presented as frequency of each genotype across all three cohorts. Odds ratio for UTI risk are presented for minor allele as odds ratio with 95% confidence interval. Comparisons were performed using Fisher’s exact test. Data from the *in vitro* experiments were normally distributed and are presented as means +/- SEM. Treated and control cells were compared by one-way analysis of variance with the Tukey procedure. Statistical analysis and
figure generation were performed using Prism GraphPad (GraphPad Software, San Diego, CA, USA).
SUPPLEMENTAL REFERENCES


