Invasion Of Vaginal Epithelial Cells By Uropathogenic *Escherichia coli*

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Abstract

Uropathogenic *Escherichia coli* (UPEC) is the primary causative agent of urinary tract infections (UTIs) and account for the majority of antibiotic prescriptions. The rise of multidrug resistant UPEC strains limits UTI treatment options, leading to more severe outcomes. In this light, outlining factors that allow for UPEC to reside within human reservoirs that contribute to recurrent UTIs (rUTIs) is of importance. Due to the shorter urethra length and perineal distance, UTIs predominately occur in woman. Several studies demonstrate vaginal colonization by UPEC precedes UTI, and the vagina is a likely reservoir for recurrent infections. Approximately 30-50% of women, experience recurrent UTIs (rUTIs). rUTIs can originate from either: 1) the re-emergence of UPEC that invaded the bladder urothelium and form quiescent intracellular reservoirs, thus evading antibiotic treatment or 2) the re-accession of UPEC from the intestinal reservoir across the perineum, vaginal introitus, and to the urethra. Previous studies have demonstrated that prior to UTI, UPEC adheres to vaginal epithelial cells (VECs). However, the full extent to which UPEC interact with epithelial cells, as it transverses the perineal space, remains largely unknown in both murine models and humans with acute or chronic UTIs. We have begun to assess the full extent to which UPEC colonizes the vaginal epithelium. Here, we show prototypical and clinical UPEC isolates adhere to VECs. Additionally, we find that UPEC invades VECs in cells in an vaginal cell line model, acute and chronic murine UTI models, as well as, VECs from clinical samples from women with a history rUTI. Our results demonstrate that UPEC invades VECs where it may reside safely from neutrophils, antibiotics, and away from the competition of the host's microbiota. We propose that UPEC invasion of VECs may serve as vaginal intracellular communities (VICs) that re-seed the occurrence of rUTI in woman.

Background

UPEC is Equipped to Thrive in Various Niches

- Unlike commensal *E. coli,* UPEC is able to transverse from the intestinal to the urinary tract.[1]
- While crossing the perineal space, UPEC colonizes the vaginal lumen and adheres to vaginal epithelial cells mediated by type 1 pili.[1,2,3]
- Once in the bladder, UPEC invades the urothelium and establishes intracellular communities and undergoing an invasion cascade.[2,3,4]

UPEC Infection Cascade Within the Urothelium

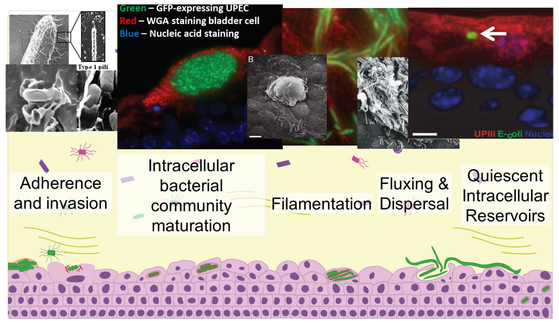
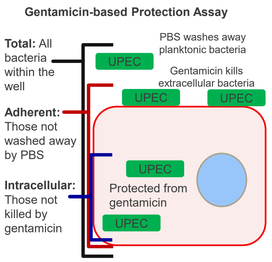


Fig 1. Diagram depicting the pathogenic cascade of UPEC in the urothelium. UPEC adheres to the urothelium and invasion is mediated by type 1 pili via zipper-like mechanism. Within bladder epithelial cells, UPEC escapes non-degradative vacuoles and establishes intracellular bacterial communities. As UPEC escapes the immune system, UPEC elongates into filaments. UPEC filaments flux outside of the urothelial cells, and disperse into individual cells in the bladder lumen. As bladder epithelial cells are exfoliated, UPEC is able to reach the transitional epithelial cells. Within this deeper layer, invading UPEC become metabolically dormant forming quiescent intracellular reservoirs (QIRs). After the passage of threats of the immune system and antibiotics, bacteria in QIRs expand and reinitiate the cycle. Images adapted from Martinez, *et al.*, Mysorekar and Hultgren, and Kostakioti, *et al.*

Hypothesis

UPEC invades vaginal epithelial cells during the course of colonizing the vaginal lumen.

Methods



Transmission and Scanning Electron Microscopy (SEM and TEM). VK2 E6/E7 cells grown in 6-well (TEM) or 12-well (SEM) tissue culture plates and infected with UTI89. Cells were washed and fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate for 30 minutes at room temperature. For TEM, specimens were placed on glow-discharged formvar/carbon-coated copper grids and stained with 1% uranyl acetate for 90 seconds. A Philips/FEI T-12 transmission electron microscope was used for sample analysis. Samples for SEM were critical point dried and placed onto aluminum stubs prior to sputter coating with gold-palladium. Images were collected for each sample and representative images were taken with an FEI Quanta 250 Field-emission gun scanning electron microscope. Images were taken from a minimum of three biological replicates.

Fluorescence microscopy. VK2 E6/E7 cells were grown on glass slides and infected with UTI89 at a MOI of 5. Were indicated paraformaldehyde-killed bacteria were treated with 3.4% paraformaldehyde (PFA) for 90 minutes prior to addition to VECs. Samples were washed with PBS and 3.4% PFA. Samples were permeabilized with Triton X-100 and washed. Where indicated, VECs were stained with wheat germ agglutinin (r-WGA) (1:500 dilution), to outline cell membranes or phalloidin (1:40 dilution), to stain F-actin, conjugated with tetramethylrhodamine. For immunofluorescence, samples were permeabilized then blocked with 2 % BSA and washed. Primary antibodies were applied in PBS with 0.1 % BSA overnight at room 4 °C with gentle rocking. Primary antibodies used in this study included rabbit a-*E. coli* antibody (1:1000) (US Biologicals), mouse a-uroplakin III antibody (1:1000) (Abcam), goat a-cytokeratin 13 antibody (1:500) (Abcam). Samples were washed once with PBS prior to secondary antibody application. Secondary antibodies were applied in PBS with 0.1 % BSA for one hour at room temperature with gentle shaking. Secondary antibodies (dilution 1:1000) used in this study were donkey a-goat, a-rabbit, and a-mouse IgG conjugated with Alexa Fluor 546, 488, and 594 respectively. ToPro3 (1:1000 dilution), a DNA specific stain, was used for counter staining for 20 minutes. All samples were washed a final three times with PBS and mounted using ProLong Diamond. Images were acquired with a LSM 710 META Inverted with plan-apochromat 63X/1.4 immersion oil objective. Post-acquisition analysis was performed using Zen and Imaris software. Images were cropped in Photoshop CC (Adobe, San Jose, CA) for display into figures.

Results

UPEC Clinical Isolates Invade Immortalized VECs

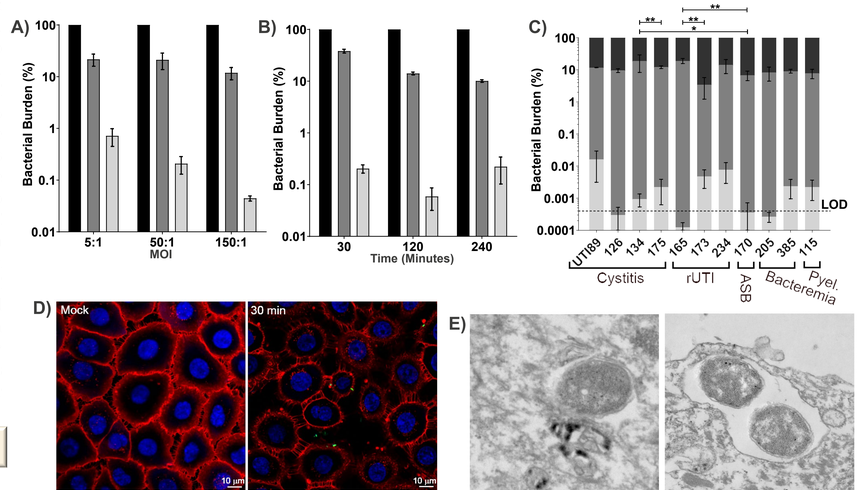
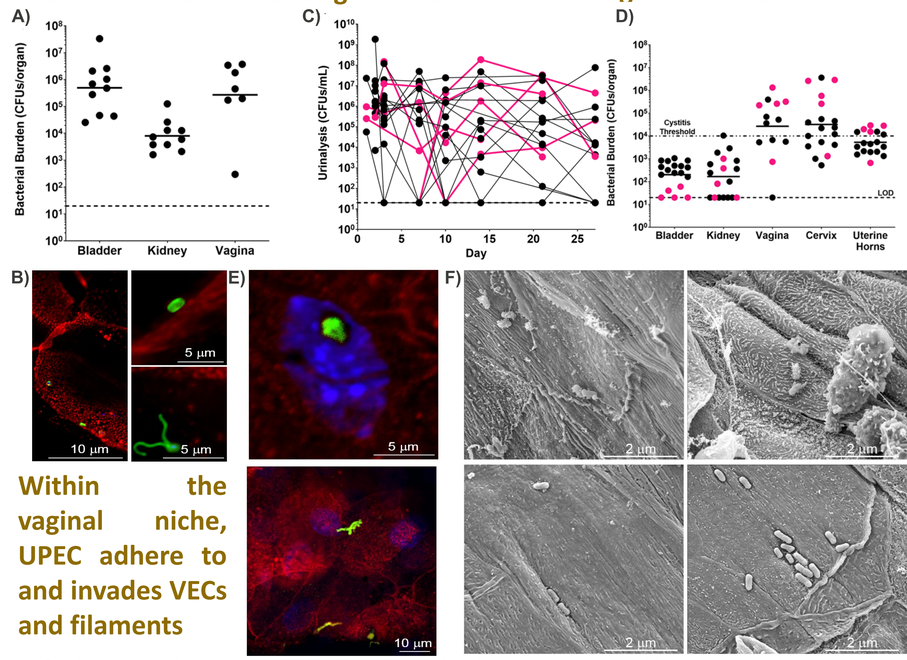


Fig 2. UPEC adheres to and invades VK2 E6/E7 VECs. VK2 E6/E7 cells were infected with the indicated strain of UPEC. **a,** and **b,** Gentamicin-based adherence and invasion assay of VECs infected by: **a,** UTI89 at the indicated MOIs for 30 minutes, **b,** UTI89 at a MOI of 5:1 for up to 240 minutes, and **c,** performed with clinical UPEC isolates for two hours. Isolates adhered to and invaded VECs to different extents. Adherent (dark gray bars) and intracellular (light gray bars) bacterial populations are relative to the average of the total population of bacteria (black bars) within the well. Data are representative of the mean of three independent experiments performed in triplicate with error bars representing s.e.m. For statistical analysis 2-way analysis of variance with Tukey post-hoc test (*$P < 0.05$, ** $P < 0.01$). **d,** Confocal laser scanning microscopy images with addition of UTI89 (pCom-GFP) for 30, 120, and 240 minutes that were stained with ToPro-3 for DNA and r-WGA to outline VEC membranes. Images are representative of three independent experiments. **e,** UTI89 at a MOI of 50:1 for two hours imaged by TEM. Images are representative two independent experiments.

UPEC Invades VEC During Colonization of the Vagina Of Mice with UTIs



Within the vaginal niche, UPEC adhere to and invades VECs and filaments

Fig 3. UPEC invades vagina epithelial cells during colonization of the reproductive tract in acute and chronic murine UTI models. C3H/HeN female mice were transuretherally infected with UTI89 in **a, b,** acute and **c-f,** chronic UTI models. **a,** Bacterial burden of an acute UTI in the bladder, kidneys, and vaginas from mice with an acute, and **b,** representative images of immunofluorescence of vaginas from three mice. Chronically infected mice **c,** urine titers **d,** bacterial burden of organs from urinary and reproductive tract. Dashed lines represent limit of detection, and dotted line represents threshold for cystitis. Pink highlight designates mice at or near the limit of detection in the bladder. Microscopy was performed on vaginal tissue of six mice from the chronic UTI model **e,** immunofluorescence and **f,** SEM. Immunofluorescence was performed with a-*E. coli* antibody and rWGA and ToPro-3 staining. Representative images from chronically infected mice are from six individual mice.

UPEC Invades Vagina Through a Zipper Mechanism

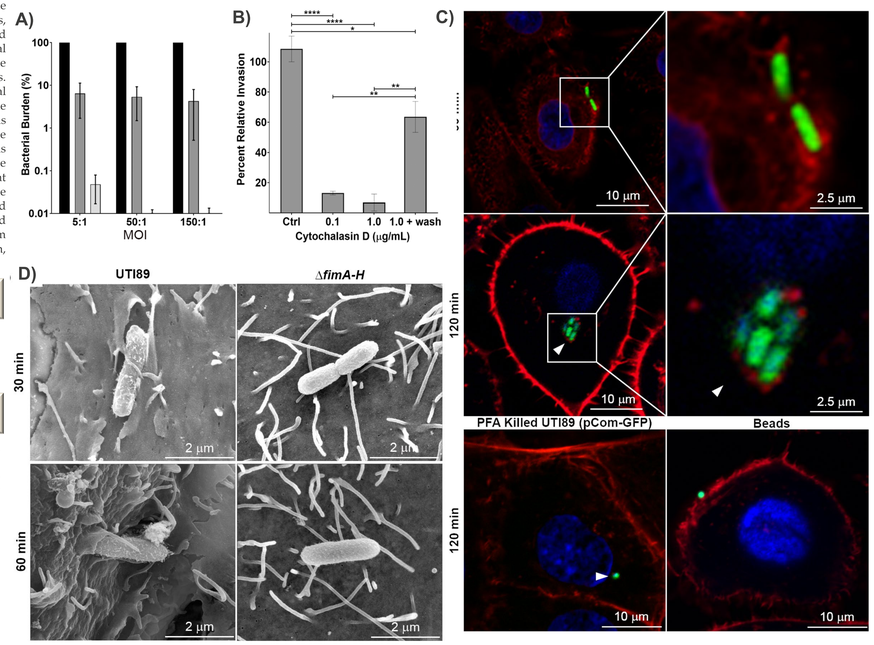


Fig 2. UPEC invades vaginal epithelial cells through a zipper-like mechanism. a, Adherence and invasion assay was performed for two hours at the indicated MOI with UTI89 Δ*fimA-H*. Adherent (dark gray bars) and intracellular (light gray bars) bacterial populations are relative to the average of the total population of bacteria (black bars) within the well. **b,** Invasion as determined by gentamicin-based invasion assay with UTI89 with the indicated level of the F-actin inhibitor cytochalasin D. Inhibition of F-actin reversibly inhibited UTI89 invasion into VEC. Percent invasion is relative to the control group. Data are representative of the mean of three independent experiments performed in triplicate with error bars representing s.e.m. For statistical analysis 2-way analysis of variance with Tukey post-hoc test (*$P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$). **c,** Adherent and invasive UTI89 (pCom-GFP), PFA-killed UTI89, or latex beads associated with VK2 E6/E7 with stained by phalloidin conjugated with tetramethylrhodamine, for F-actin, and ToPro-3. Red bundles specifically around bacteria indicated actin polymerization occurs prior to invasion **d,** SEM images of UTI89 and UTI89 Δ*fimA-H* interacting with the surface of VK2 E6/E7 cells. SEM images of the subtle envelopment of UTI89 by VEC membranes is consistent with a zipper-like model of invasion. Images are representative of three independent experiments.

UPEC Invades VECs of Woman with a History of rUTIs

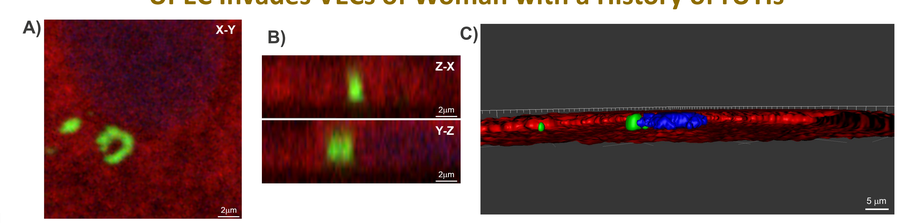
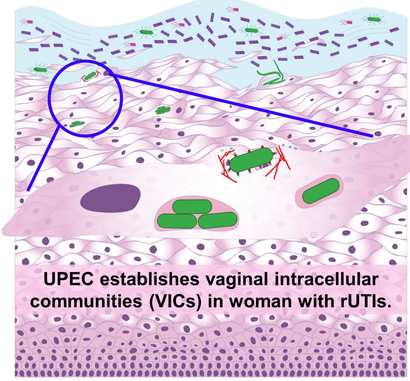


Fig 4. UPEC invades the vaginal epithelial cells of women with a history of rUTIs. Immunofluorescence microscopy was used to search for *E. coli* within VEC collected by vaginal swabbing of women with a history of rUTIs. **a-c,** Samples were stained with a-*E. coli* (green), a-uroplakin III (red), and a-cytokeratin 13 (yellow) and ToPro-3 (blue). **a,** The XY-plane image from a Z-stack series of a VEC with *E. coli* surrounded by the VEC specific cytokeratin 13. Ortho views of **b,** YZ-plane and ZX-plane reconstructed from the Z-stack series images showing *E. coli* surrounded by cytokeratin 13 within a VEC near the nucleus. **c,** Surface projections of *E. coli* (green), VEC cytokeratin 13 (red), uroplakin III (yellow), and nucleus (blue) generated in Imaris. Imaris software was used image analysis of the Z-stack.

Conclusions

1. UPEC invades vaginal epithelial cells.
2. UPEC invades vaginal epithelial cells through a zipper-like mechanism.
3. UPEC persists in vaginal epithelial cells of women with rUTIs.



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Funding: APS 1-04-520-9211 to M.H., NIH R01 AI107052-01A1 to M.H., MicroVU TIPS to J.B.