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A Murine Model for *Escherichia coli* Urinary Tract Infection

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Abstract

Urinary tract infections (UTI) are among the most common bacterial infections of humans. The mouse provides an excellent and tractable model system for cystitis and pyelonephritis caused by *Escherichia coli* and other uropathogens. Using a well-established model of experimental cystitis in which the bladders of female mice are infected via transurethral catheterization, the molecular details of the pathogenesis of bacterial cystitis have been substantially illuminated in the last decade. Uropathogenic *E. coli* attach to bladder epithelium (both in human and mouse) via adhesive type 1 pili, establish a replicative niche within epithelial cell cytoplasm, and form intracellular bacterial communities that are protected from antibiotic effects and immune clearance. The use of different inbred and mutant mouse strains offers the opportunity to study outcomes of infection, including resolution, formation of quiescent intracellular bacterial reservoirs, chronic bacterial cystitis, and recurrent infections. Urine, bladder, and kidney tissues can be analyzed by bacterial culture, histology, immunohistochemistry, immunofluorescent and confocal microscopy, electron microscopy, and flow cytometry, while a broad array of soluble markers (e.g., cytokines) can also be profiled in serum, urine, and tissue homogenates by ELISA, Western blotting, multiplex bead array, and other approaches. This model promises to afford continued opportunity for discovery of pathogenic mechanisms and evaluation of therapeutic and preventive strategies for acute, chronic, and recurrent UTI.

Keywords

Urinary tract infection; Cystitis; *Escherichia coli*; Intracellular bacterial community; Bacterial persistence

1 Introduction

The human urinary tract is among the most common sites of bacterial infection. Uncomplicated urinary tract infections (UTIs) in the USA result in >10 million medical visits and nearly \$4 billion in medical expenditures annually [1]. Predisposing factors for UTI include structural and urodynamic abnormalities, pregnancy [2], diabetes [3], bladder catheterization [4], prostate enlargement [1], HIV infection [5], and sexual activity [6, 7]. Notably, most outpatient UTIs occur in otherwise healthy women with no identifiable risk factors. Indeed, 50 % of women will experience a UTI during their lifetimes, and up to 25 % will suffer recurrence of infection within 6 months following treatment of initial UTI [8]. Two-thirds of these recurrences are attributable to the initial strain recovered from a given patient [8, 9].

Uropathogenic *Escherichia coli* (UPEC) cause the bulk of all diagnosed UTIs [8] and are the leading cause of both community-acquired and healthcare-associated UTIs. UPEC wield distinct virulence determinants to cause disease in the bladder (cystitis) and in the kidney (pyelonephritis). Pyelonephritis is distinguished clinically from cystitis by the presence of flank pain, fever, nausea, and vomiting. It follows that a proportion of patients diagnosed with cystitis may also have bacterial colonization of the kidney(s) that does not elicit the overt symptoms typically associated with pyelonephritis. Because no routine clinical testing clearly defines which urinary tissues are involved in an infection, classification of tissue tropism for each clinical isolate relies on patient symptom histories. As a result, it can be difficult to ascertain specific bacterial virulence traits that associate only with cystitis or pyelonephritis.

In the last 1–2 decades much has been learned about the initiation, progression, and resolution of mammalian UTIs using the murine model which will be described herein. In short, female mice of various backgrounds (with varying susceptibility to the establishment of UTI) can be temporarily anesthetized and UPEC (or other uropathogenic species) can be delivered to the bladder by means of catheterization. Following the procedure, which in experienced hands requires only 2–3 min per mouse, animals can be monitored clinically and by collection of blood and urine. Mice are sacrificed at designated time points following infection, and the body fluids (blood and urine) and organs (kidneys and bladder) are harvested and can be processed for numerous cellular and soluble analyses (e.g., bacterial loads, tissue microscopy and histology, leukocyte populations, tissue and serum cytokines).

The mouse represents a desirable model system for mammalian UTI, as the bladder structure and cellular composition mimic those found in the human bladder. As in human disease, bacterial attachment to the bladder epithelium is mediated by a heteropolymeric bacterial surface fiber known as the type 1 pilus [10], while a related organelle (the P pilus) can promote UPEC binding to globoseries glycolipids on the kidney epithelial surface [11]. Recent work in this UTI model has demonstrated that UPEC not only bind, but also invade superficial bladder epithelial cells [12–27] and kidney epithelial cells [28, 29]. Invasion into bladder epithelial cells during cystitis has also been observed with other common uropathogens, including *Staphylococcus saprophyticus* [30], *Klebsiella pneumoniae* [31], and *Salmonella enterica* [22]. This process entails the uptake of UPEC into Rab27b-positive fusiform vesicles, which are normally shuttled into and out of the apical membrane to regulate bladder surface area during the accumulation of urine. As a further result of this vesicle traffic, a proportion of internalized bacteria are returned to the luminal space via exocytosis [22, 23]. A minority of internalized UPEC then gain unrestricted access to the cytoplasm and grow exponentially in coccoid form, forming clonal, biofilm-like intracellular bacterial communities (IBCs) that can contain up to 10^5 organisms and cause superficial epithelial cells to protrude into the bladder lumen [18]. Arriving phagocytes (largely neutrophils) hone to infected cells but cannot access the replicating bacteria within [32]. Subsequently, bacteria at the IBC periphery regain a bacillary shape or even a phagocytosis-resistant filamentous form [32–34], exit the infected cell, and proceed to infect naïve epithelial cells or to leave the host via micturition. Meanwhile, infected epithelial cells may undergo apoptosis and also be eliminated in the urine [35–38]. Overall, the IBC niche

provides UPEC with a protected haven for replication in the face of a burgeoning soluble and cellular inflammatory response. Underscoring the importance of this stage in the establishment of bacterial cystitis, UPEC also employs multiple strategies to dampen initial neutrophil recruitment (see below), allowing a window of opportunity for epithelial invasion and establishment of the intracellular niche. IBC formation might also serve to select subpopulations of introduced UPEC that possess characteristics optimized for intracellular life, subversion of immune effectors, and intraepithelial persistence (see below). Finally, the relevance of the murine IBC cascade to human cystitis is corroborated by the identification of filamentous UPEC (and other Gram-negative uropathogens) and exfoliated, IBC-bearing uroepithelial cells in the urine of patients with UTI [39, 40].

The catheterization model of murine UTI has also been used extensively to characterize the inflammatory response to UPEC arriving in the bladder and kidneys, and to elucidate mechanisms by which UPEC manipulates host responses (reviewed in [41]). To summarize here, UPEC components, including lipopolysaccharide and pili, ligate Toll-like receptors (primarily TLR4) on host epithelium and resident leukocytes [42], stimulating NF- κ B and other signaling pathways, eliciting the local secretion of inflammatory cytokines (e.g., IL-1, IL-6, IL-8) [43, 44], and drawing neutrophils to the infected tissues. These findings in mice correlate with genetic polymorphisms in TLR4 and IL-8 receptors that have been demonstrated in selected human populations with repetitive UTI [45-49]. On the bacterial side, UPEC has been demonstrated to downregulate the production of pro-inflammatory cytokines by uroepithelial cells [50-54], deliver leukotoxins [55,56], and attenuate the trafficking of neutrophils across the uroepithelium [57-59]. These studies represent only a fraction of published work that demonstrates the broad utility of the murine UTI model in dissecting the host-pathogen conversation during bacterial cystitis and pyelonephritis.

Finally, recent advances have revealed multiple phenotypes of UPEC persistence within the bladder. In C57BL/6 mice, bladder infection following inoculation via catheter resolves in nearly all animals within 2 weeks of infection, leaving behind a quiescent intracellular reservoir of UPEC [19] that manifests as small nests of 4–8 organisms visible in Lamp1-positive vesicles within bladder epithelial cells [60]. These bacteria resist clearance by systemically administered antibiotics, appear not to attract immune attention, and reemerge periodically in the murine host to yield recurrent bacteriuria [61]. More recent studies in C3H mice demonstrate a “bimodal” phenotype in which some animals resolve acute infection, while others develop chronic, high-titer cystitis with ongoing cellular inflammation. This model has already yielded novel, translatable insights into host immunologic determinants of the outcome of acute infection and propensity for recurrence [62, 63], as will be addressed further in the methods outlined below.

2 Materials

1. Luria-Bertani (LB) broth: Dissolve 10 g tryptone, 5 g yeast extract, and 10 g NaCl in 1 L deionized (dI) water, and autoclave for 30 min at 121 °C.

2. LB agar plates: Add 16 g agar powder to 1 L LB medium. Autoclave 30 min at 121 °C and allow the medium to cool to 50–60 °C. Pour approximately 25 mL LB agar into 100 × 15 mm plastic petri dishes.
3. Phosphate-buffered saline (PBS): 800 mL of distilled water, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, 0.24 g of KH₂PO₄, adjust the pH to 7.4 with HCl, add distilled water to a total volume of 1 L.
4. Dulbecco's PBS for tissue culture: 2.7 mM KCl, 1.5 mM KH₂PO₄, 137.9 mM NaCl, 8.1 mM Na₂HPO₄·7H₂O.
5. Triton X solution: 0.01 % (v/w) Triton X-100 in PBS.
6. Sterile lubricating jelly.
7. Ethanol 70 %: 70 % (v/v) ethanol (denatured).
8. Paraformaldehyde 20 % (w/v) solution: Electron microscopy grade.
9. Neutral-buffered formalin solution 10 % (w/v).
10. ProLong Gold antifade reagent.
11. Isoflurane.
12. Mobile rodent anesthesia induction system: Respirator including an isoflurane vaporizer with reservoir, a nose cone and/or induction chamber, and waste anesthetic gas-scavenging canisters.
13. Intramedic non-radiopaque polyethylene tubing, PE10 [inner diameter 0.28 mm (0.011 in.), outer diameter 0.61 mm (0.024 in.)].
14. 30-Gauge hypodermic needles, 1/2 in.
15. 1 mL tuberculin Slip-Tip syringe.
16. Rotor-stator tissue homogenizer with 0.7 mm × 95 mm sawtooth generator probe, or FastPrep24 bead beater with 2 mL screw-top tubes and 5 mm stainless steel beads.
17. SYLGARD 184 silicone elastomer kit.
18. 6-Well polystyrene flat-bottom culture plates.
19. 96-Well polystyrene round-bottom microplates.
20. Sterile microcentrifuge tubes.
21. 5 mL Falcon polycarbonate tubes.
22. Minutien pins, 0.2 mm base diameter.
23. Fisherbrand TRUFLOW tissue and biopsy cassettes.
24. X-gal solution: 25 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in dimethylformamide.

25. LacZ wash buffer: 2 mM MgCl₂, 0.01 % sodium deoxycholate, 0.02 % Nonidet-P40 (Roche) in PBS, pH 7.4.
26. LacZ stain: Mix 9.5 mL LacZ wash buffer with 0.4 mL 25 mg/mL X-gal (final concentration 1 mg/mL) and 0.1 mL combined stock solution of 100 mM potassium ferrocyanide and 100 mM potassium ferricyanide (final concentration 1 mM each).
27. Digestion buffer: 1 mg/mL collagenase D or IV plus 100 µg/mL DNase I in PBS.
28. Confocal and/or epifluorescent microscopes.

3 Methods

3.1 Preparing the Bacterial Inoculum

1. Inoculate 20 mL Luria-Bertani (LB) broth with a single colony of UPEC or from a frozen bacterial stock (*see* Note 1). Grow statically for 18–24 h at 37 °C.
2. Subculture 20 µL of overnight culture into 20 mL fresh LB broth and grow statically for 18 h at 37 °C.
3. Centrifuge culture at room temperature for 10 min at 3000 × *g*, discard supernatant and resuspend pellet in 10 mL sterile PBS.
4. Normalize OD₆₀₀ of a 1:10 dilution of the washed culture to 0.35 (for 1 cm path length). For the UPEC strain UTI89 grown under the above conditions, this corresponds to a titer of 1–2 × 10⁷ colony-forming units (CFU) per 50 µL inoculum (*see* Note 2).
5. Verify titer inoculum by plating serial dilutions using the spotting method (*see* Subheading 3.2).

3.2 Bacterial Enumeration Using the Spotting Method

1. Use sterile, round-bottom clear polystyrene 96-well plate (Corning 3788).
2. Fill rows A-F with 180 µL sterile PBS per well, where each column allows for serial dilution of a single sample.
3. Add 20 µL of the inoculum to the top row (row A) to make a 1:10 dilution and mix by gently pipetting up and down; Discard pipette tips.

¹For long-term studies involving urinalysis or challenge infections, the use of a chromosomally encoded antibiotic marker (e.g., conferring resistance to kanamycin or chloramphenicol) is recommended to distinguish the infecting UPEC strain from contaminating flora of the skin and vagina and from the initial infecting strain during challenge infections. The use of spectinomycin-resistant strains can be problematic, as many spectinomycin-resistant bacteria reside in the vaginal flora. If spectinomycin is used for selection, the use of MacConkey agar containing spectinomycin may be necessary to eliminate contamination from spectinomycin-resistant vaginal flora. The presence of antibiotics may alter bacterial expression of virulence factors even if the strain is able to replicate. Therefore, in preparing the inoculum it is best to grow the bacteria without antibiotics during static growth in LB broth. However, antibiotic selection may be used when streaking on LB agar for single colonies from freezer stock.

²For a tenfold higher inoculum (~10⁸ CFU), apply the same dilution factor to undiluted washed culture. The relationship between culture absorbance (OD₆₀₀) and viable bacterial titer can vary with bacterial strain and culture/laboratory conditions and should not be assumed to be identical to those described in Subheading 3.1.

4. Aspirate 20 μ L from row A and add to row B to make another 1:10 dilution. Mix by gently pipetting. Discard tips.
5. Repeat **step 4**, aspirating from row B to row C, etc., until row F contains sample diluted 1:10⁶ in PBS.
6. Using a repeating multichannel pipettor, aspirate 10 μ L of each row for a single column and spot onto an LB agar plate, with or without antibiotic. Repeat four additional times so that a column is spotted five times on a single plate, 50 μ L ($5 \times 10 \mu$ L) total per dilution.
7. Allow plates to dry face up, then invert, and incubate overnight at 37 °C (*see* Note 3).
8. Count colonies for the row (dilution) that contains a total of 15–150 CFU and calculate the overall titer.
9. Urine and bladder or kidney tissue homogenates can be titered in a similar fashion (*see* Note 4).

3.3 Preparing Urinary Catheters

1. Urinary catheters are best prepared in a sterile laminar flow hood equipped with an ultraviolet lamp (such as that used for tissue culture), and all working surfaces, gloves, and instruments should be sprayed with 70 % ethanol.
2. With a sterile blade or scissors, cut a length of polyethylene tubing (PE-10) sufficient for the number of catheters being made.
3. Place a sterile 30-ga $\times$ ½-in. needle on a sterile 1 mL tuberculin syringe.
4. Pick up one end of the polyethylene tubing with forceps and gently slide it onto the needle up to the hub.
5. Cut the tubing approximately ½ in. from the needle tip, remove the catheter from the syringe, and place it in a sterile petri dish.
6. Repeat **steps 2–5** as required. A minimum of one catheter is needed per cage of five mice to be infected.
7. UV-irradiate catheters for at least 30 min to ensure sterility.
8. Replace and secure the lid of the petri dish and store catheters for future use.

³UPEC grow well on LB agar plates at both room temperature and 37 °C, so the time allowed for drying is not critical. However, this is not true for some other media. Therefore, the use of selective media agar plates to eliminate contamination from the skin and vaginal flora may require more strict culture conditions for bacterial enumeration. For example, UPEC grow poorly on MacConkey agar at room temperature and thus the plates must be dried quickly and placed into 37 °C promptly after plating or bacterial numbers may be underestimated.

⁴For longitudinal urinalysis, because of the inherent imprecision in quantifying urine titers due to variability in urine collection, it may be advisable to spot only 10 μ L per sample, to allow urines from 5 to 6 mice to be titered using a single plate, thus saving on materials. For tissue titers, PBS is typically not added to row A; instead, undiluted homogenates are placed in this row for serial dilution.

3.4 Intravesical Inoculation of Mice with Bacteria

1. Draw up the bacterial inoculum (Subheading 3.2) into a tuberculin syringe with 10 μ L gradations.
2. Place catheter on syringe and expel all air.
3. Trim catheter to 1–2 mm past the tip of the catheter needle, using fine scissors sterilized with 70 % ethanol or alcohol swabs.
4. Set syringe down, with catheter tip in sterile lubricating jelly.
5. Induce anesthesia in mice with 2–3 % isoflurane using a vaporizer and induction chamber; be careful not to overcrowd the chamber with mice (*see* Note 5).
6. Once mice are anesthetized, remove a mouse and place it supine on the working surface (absorbent pad or piece of Styrofoam covered with a clean paper towel) with the tail directed toward the technician; maintain anesthesia using a nose cone assembly connected to the vaporizer (*see* Note 6).
7. Place a finger on the caudal abdomen, locate the bladder, and apply pressure in a gentle rostral/caudal motion to empty the bladder of urine (*see* Note 7).
8. Place the lubricated catheter tip into the urethral opening, with the catheter and syringe oriented perpendicular to the table.
9. Advance the catheter about 0.5 cm or until resistance is encountered, and then rotate the syringe caudally in an arc 90° so that it is now parallel to the table and tail. The catheter should now be free to pass through the pelvis and into the urinary bladder (*see* Note 8).
10. Once the catheter is advanced to the hub of the needle, slowly inject the bladder with 50 μ L of inoculum, delivering it in 10 μ L increments over 10–15 s to allow the bladder to slowly expand (*see* Note 9).
11. Remove the syringe/catheter assembly and set aside with catheter tip in lubricating jelly.

⁵Anesthesia can be safely maintained with 2–3 % isoflurane for an extended period of time, provided that the oxygen flow rate is sufficient (this will vary with vaporizer circuit dead space and frequency of induction chamber opening). Alternatively, anesthesia can be induced one mouse at a time in a glass jar containing a steel tea ball that has been filled with cotton balls soaked with isoflurane. However, mice should be monitored very closely and removed immediately from the glass jar after induction (when the respiratory rate begins to decrease) to avoid accidental overdosing and death.

⁶If a nose cone connected to a vaporizer is not available, a 50 mL conical tube containing isoflurane-soaked cotton balls packed into the bottom may be used. In this case, the concentration of isoflurane that the mouse is inhaling can be crudely adjusted by altering how deeply the head is positioned in the conical tube.

⁷Emptying the bladder of urine prior to bacterial inoculation is a critical step in reducing variability during experimental infection.

⁸If resistance is encountered when passing the catheter through the internal urethral sphincter, re-lubricate the catheter and try again. If still encountering resistance, arc the syringe from side to side while gently applying forward pressure. The catheter should pass easily into the bladder—it should never be forcibly passed into the bladder, as this may result in trauma to the lower urinary tract and perhaps death. If the catheter cannot be advanced within 30 s, the technician should stop and move on to the next mouse, coming back a little bit later to the mouse that is causing difficulty. When first learning intravesical inoculation technique, it is a good idea to do practice inoculations with a dye and then dissect out the bladder and urethra to examine for signs of trauma or puncture.

⁹Excessive volume or too rapid instillation can lead to iatrogenic vesicoureteral reflux (VUR) in mice, causing the inoculum to be forced into the upper urinary tract. In host strains genetically resistant to VUR, the incidence of kidney infections increased as the volume of UPEC suspension instilled into the bladder exceeded 50 μ L [64, 65]. In contrast, C3H and CBA mice are genetically susceptible to VUR, and kidney infection occurs in all mice at inocula of 10^7 and 10^8 CFU per 50 μ L.

12. Record mouse identification so that each individual can be tracked for longitudinal analyses. Use an ear punch or ear tags to identify co-housed mice. Replace the inoculated mouse back in its cage to recover.
13. Repeat **steps 6–12** for each mouse, changing catheters as needed (e.g., every 5 mice). If different bacterial strains or mutants are part of the same experiment, catheters should be changed between strains.

3.5 Longitudinal Urinalysis

1. For long-term infections in mice, the course of infection can be monitored longitudinally by collecting and analyzing their urine over time (*see* Note 10).
2. At each time point, grasp the mouse at the base of the tail and remove from the cage.
3. In one hand, set the mouse on the edge of the cage wire rack with the tail pointing toward the technician while keeping hold of the tail by the thumb and forefinger. In the other hand, hold an open, sterile, pre-labeled 1.5 mL microcentrifuge tube.
4. With the hand that is holding the mouse, use the remaining fingers to apply gentle pressure to the back of the mouse, so that the edge of the wire rack exerts pressure on the ventral suprapubic region of the mouse. Collect the urine in tube.
5. Bacterial enumeration of urines can be performed as explained in Subheading 3.2.
6. Urine white blood cell enumeration can be performed quantitatively using a manual hemacytometer (*see* Note 11).
7. An aliquot of urine can be centrifuged at $>12,000 \times g$ for 5 min and the supernatants stored at -20°C or -80°C for future immunological studies, such as urine cytokine and/or immunoglobulin quantification.

3.6 Antibiotic Treatment and Bacterial Challenge Studies

1. Mice with chronic cystitis (*see* Note 12) can be treated with antibiotics to eliminate infection by susceptible organisms at any time after experimental inoculation.

¹⁰For long-term infections (2–4 weeks), it is sufficient to collect urines at days 1, 3, 7, 10, 14, and weekly thereafter. Persistent high-titer ($>10^4$ CFU/mL) bacteriuria at every time point over 4 weeks is a sensitive and specific indicator of ongoing bladder infection (i.e., chronic cystitis) [62].

¹¹Alternatively, semiquantitative urine neutrophil analysis can be performed by sedimenting a standard volume of urine (8 μL diluted in sterile PBS is a convenient volume, as 80 μL of 1:10 diluted urine is typically what remains in row A after plating on both LB and LB with an antibiotic for bacterial titers) onto glass slides using a cytocentrifuge (e.g., Wescor CytoPro 7620 at $440 \times g$ for 6 min with high acceleration). Stain the slides using Hema-3, Diff-Quik or similar cytology stain, and score degree of pyuria based on cells with segmented nuclei (neutrophils) per high-power field (hpf; 400) (0: $<1/\text{hpf}$; 1: 1–5/hpf; 2: 6–10/hpf; 3: 11–20/hpf; 4: 21–40/hpf; and 5: $>40/\text{hpf}$). Ghosts (degraded cells without distinct nuclei) are not counted.

¹²Some mouse strains (e.g., C3H/HeN) are susceptible to chronic and recurrent infections. It has been demonstrated that the outcome of initial infection in naïve animals can alter their susceptibility to subsequent challenge infections [62]. To specify the outcome of infection, longitudinal urinalysis can be performed for 4 weeks as described in Subheading 3.5. High-titer persistent bacteriuria ($>10^4$ CFU/mL at every time point) is a strong predictor of chronic bacterial cystitis with UPEC, whereas having any time point with bacteriuria $<10^4$ CFU/mL is a strong predictor of resolution of bladder infection. PBS mock-inoculated mice are useful here to provide age-matched naïve controls for bacterial challenge.

2. Add commonly available veterinary formulations of trimethoprim/sulfamethoxazole to the drinking water at concentrations of 270 and 54 µg/mL, respectively, for 3 or 10 days [61].
3. Antibiotic water should be changed at least every other day, as needed.
4. Monitor urines weekly for bacteriuria to ensure sterilization of urine (*see* Note 13).
5. Four weeks or more after initiation of antibiotic therapy, mice can be challenged with UPEC or other bacteria by intravesical inoculation, preferably using a bacterial strain that can be differentiated from the initial infecting strain by phenotypic selection (e.g., an antibiotic marker).

3.7 Tissue Harvesting

1. Humanely euthanize the mouse by CO₂ asphyxiation or cervical dislocation under anesthesia.
2. Place the mouse on the back and spray the ventral abdomen with 70 % ethanol.
3. Using blunt surgical scissors and fine forceps, pull up the skin of the caudal ventral abdomen with the forceps and make a small cut through the skin and peritoneum.
4. Once the peritoneal cavity is entered, use the scissors to make a V-shaped opening in the abdomen by cutting toward the ribs on either side of the body.
5. Manually pull the flap forward toward the nose (rostrally) to bluntly make the opening larger.
6. Remove bladder, kidneys, and other tissues desired for downstream analyses, e.g., tissue homogenization for bacterial enumeration and cytokine analysis (Subheading 3.8), flash freezing of bladder tissue in liquid nitrogen for transcriptomics and proteomics studies, generation of single-cell suspensions for flow cytometry (Subheading 3.9), or fixation of tissues for imaging (Subheading 3.10).

3.8 Tissue Homogenization

1. Place bladder tissue in 1 mL PBS (0.8 mL PBS for kidneys) in a 5 mL Falcon tube on ice and homogenize tissue using a rotor-stator homogenizer for about 40 s (*see* Note 14).
2. Take an aliquot of homogenate for bacterial enumeration, described in Subheading 3.2.

¹³Antibiotic therapy is generally successful in eliminating susceptible urinary tract infections, especially with antibiotics that concentrate in the urine, such as trimethoprim/sulfamethoxazole. However, some mouse strains are susceptible to abscess formation when the kidneys become infected, and treatment failures can result from poor antibiotic penetration of the abscess.

¹⁴Alternatively, tissue can be homogenized by bead beating. Place tissue in a 2 mL screw-top tube (selected according to bead-beating equipment) containing 200 µL PBS and either one (for kidneys) or two (for bladder) 5 mm stainless steel beads. Homogenize tissue by pulsing twice for 60 s at 4.0 m/s and add 800 or 600 µL PBS to bladder and kidney homogenates, respectively, to reach a final volume of 1 mL.

3. The remaining homogenate can be cleared by centrifugation at $>12,000 \times g$ for 5 min and the supernatants and cell pellets separated and frozen at -80°C for cytokine analysis and Western blotting, respectively.

3.9 Bladder Single-Cell Suspensions for Flow Cytometry

1. Bisect bladder (if desired, rinse in PBS to remove cells and debris from lumen).
2. Digest bladder tissue for 60–90 min at 37°C in 1 mL digestion buffer (1 mg/mL collagenase D or IV and 100 $\mu\text{g/mL}$ DNase I (from 1000 $\times$ stock) in PBS).
3. Pass digested tissue through a 40 μm cell strainer into a 50 mL conical tube on ice. Use the black (rubber) end of a sterile 1 mL syringe plunger to assist passage. From this point on, keep cells on ice.
4. Wash the filters twice in 1 mL PBS, and transfer the cell suspension to microcentrifuge tubes; centrifuge at $300 \times g$ for 5 min. Remove supernatant carefully with gel-loading tips.
5. Wash cells twice by resuspending in 1 mL PBS, centrifuging at $300 \times g$ for 5 min, and removing supernatants with gel-loading tips.
6. Cells can now be stained as required by the experiment (*see* Note 15).

3.10 Long-Term Fixation of Tissues for Imaging

1. Aseptically remove the tissue and place in choice of fixative (*see* Subheading 4 for types of fixation).
2. Wash fixed tissues three times in 70 % ethanol and store in 70 % ethanol in watertight container.
3. Place tissues in cassette and submit to histology services for paraffin embedding, sectioning, and hematoxylin and eosin staining (*see* Note 16).

3.11 LacZ Staining and Confocal Imaging of Bladder Whole-Mount tissue

1. Aseptically remove each bladder, bisect sharply, and pin out each hemisphere, lumen side up, on silicone elastomer surfaces in 6-well plates containing 1 mL sterile PBS, using two pairs of forceps to manipulate and stretch the bladder and to place pins (*see* Note 17).
2. Wash bladders lightly once with PBS.

¹⁵This isolation protocol is optimized for immune cell recovery. Cells can be processed for surface marker and/or intracellular staining. For surface marker staining, it is preferable to stain unfixed cells and gate out dead cells using live/dead stains such as propidium iodide or 7-AAD to improve the quality of analysis. Dead cells predominantly represent non-immune cells and can nonspecifically stain with antibodies.

¹⁶Extra unstained slides may be prepared for later immunofluorescence staining. QIRs can be quantified at >2 weeks post-infection by systematically analyzing by immunofluorescence serial sections through the bladder that have been stained with fluorescent antibodies to *E. coli* and Lamp-1 [60, 66]. A collection of 4–12 organisms within a Lamp-1-positive vesicle constitutes a QIR.

¹⁷The use of fluorescent bacteria, particularly UPEC-expressing green fluorescent protein from a multicopy plasmid [67], greatly enhances the identification of UPEC by confocal microscopy in mounted bladder halves.

3. Fix the bladders with 3 % paraformaldehyde in PBS at room temperature for 45–60 min with very gentle movement on a rotator (*see* Note 18). If visualizing the bladder by confocal microscopy, proceed with **step 4**; if only visualizing IBCs, skip to **step 9**.
4. Permeabilize the tissue with 0.01 % Triton X-100 in PBS for 10 min.
5. Stain bladders with a nuclear stain (e.g., Topro3, DAPI, or Syto 61) in 0.01 % Triton X-100 in PBS for 3–10 min.
6. Wash in fresh PBS three times for 5 min each.
7. Remove salt by washing in distilled H₂O for 5 min.
8. Mount on glass slide using ProLong Gold antifade reagent with a cover slip, and image using a confocal microscope. If proceeding to LacZ staining, bladders can be stored overnight in PBS at 4 °C.
9. Wash bladders with LacZ wash buffer, three times for 5 min each with gentle rotation.
10. Incubate in LacZ stain for 8–16 h at 30 °C in a light-shielded environment. Monitor staining at 4–6 h and continue stain incubation until punctate staining is visible (on positive control bladders infected with virulent UPEC at known time points). Overlong incubations will result in background staining of uroepithelial cells.
11. Wash in PBS three times for 5 min each.
12. Observe bladders on a dissecting scope and enumerate IBCs. It is advisable to save an image of each bladder half for quantifying IBCs and as a permanent data record.

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¹⁸Alternatively, bladders can be fixed in glutaraldehyde for scanning or transmission electron microscopy, using protocols optimized for your imaging equipment or facility.

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