VAGINAL INOCULATION OF UROPATHOGENIC *E. COLI* DURING ESTRUS LEADS TO GENITAL AND RENAL COLONIZATION

A Thesis

by

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ABSTRACT

Urinary tract infection (UTI) is one of the most prevalent bacterial infections, particularly in women, children, and the elderly. Uropathogenic E. coli (UPEC) is the predominant etiological agent of UTI. To better understand this bacterial infection, the use of the murine model has been predominately applied. In the widely-used murine model of UTI, uropathogens are directly instilled into the urinary bladder, bypassing the lower urogenital tract. We assessed whether vaginal inoculation of UPEC led to UTI and how stages of the estrous cycle would impact bacterial colonization in mice. Mice in proestrus, estrus, metestrus, and diestrus were identified by vaginal cytology and inoculated with UPEC in the vaginal tract. Bacterial loads in the urogenital tract, liver, and spleen were enumerated. Mice in estrus exhibited the highest UPEC burden in all organs except the bladder. Vaginal inoculation resulted in bladder colonization in a UPEC strain-specific manner. In contrast, transurethral inoculation of UPEC led to bladder colonization. Importantly, inoculation by both routes led to vaginal and uterine colonization and concomitant systemic dissemination to the spleen and liver. In summary, vaginal inoculation of UPEC in mice during estrus represents a novel approach to investigating infection of the kidneys and genital tract as well as systemic dissemination from the urogenital tract. Our findings suggest that estrogen primes the urogenital tract to create a conducive milieu for UPEC colonization.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background and Public Health Concerns

For decades scientists, physicians, epidemiologists and other public health officials have utilized their expertise to uncover the etiology of many new and existing infectious diseases. It is the responsibility of those individuals to protect and serve populations both domestically and globally from harmful agents that can affect both animals and humans. Dr. Bernadette Dunham, former Director of FDA's Center for Veterinary Medicine was once quoted saying:

> "No one discipline or sector of society has enough knowledge and resources to prevent emergence or resurgence of diseases in today's globalized world. Through mutual collaborations, veterinarians and physicians can accomplish so much more to advance the health of humans and animals." (103)

With the help from all areas of public health, scientific research and collaboration, we can continue to conquer some of the most harmful infectious diseases that have emerged throughout history.

Worldwide, hospitals treat patients daily who are experiencing illnesses that, in many cases, are disease-related. Those hospitalizations can range from non-infectious to infectious causes of disease and may vary based on the patient's age, sex, and race. A shared commonality is that all patients seeking medical attention hope to receive treatment and a resolution to their problem. Of those hospitalizations, over 10.5 million are bacterial. Public health statistics indicate that while bacterial infections are a principal diagnosis in emergency room visits or outpatient

clinics, many infections remain unreported. In fact, there are many individuals that do not present to a hospital for therapy and treatment. Many patients that do not seek appropriate treatment for some common infections will continue to face intermittent and recurring symptomatic illnesses. One of the most common bacterial infections seen in both men and women, with over 150 million cases per year globally, is urinary tract infections (1).

Overview of Urinary Tract Infections and *Uropathogenic E. coli*

Urinary tract infections (UTIs) have many clinical forms, often affecting many different populations. A UTI is typically defined as an inflammatory response associated with bacteriuria of the upper, lower, or entire urinary tract, which is initiated to help fight off the infection within the body (8, 60). UTIs have been observed in patients with spinal cord injuries, as well as from those who have been exposed to sexually transmitted infections, diabetes, and, more commonly, urethral catheterization. Males have also been reported to contract urinary tract infections; however, male UTIs are less common compared to females and occur at less than 5-8 infections per 10,000 a year (1). While men can become infected, the population that is most commonly and heavily impacted by UTIs is women. Bacterial UTIs are typically seen in older women. However, they can also negatively impact young children and young adult women. Estimates show that one-third of young adult women are diagnosed with a UTI before they are 24 years old and can suffer the burden of a urinary tract infection once, twice, or even three times a year (61). This substantially affects their day-to-day lives, mental health, and sense of well-being. More than 50% of UTI patients will suffer from clinical depression, while 38.5% will suffer from anxiety (60). UTIs cause an unnecessary burden for all who experience them, especially women. This critical public health concern is why we must search for new insights for better prevention, diagnosis and treatment for UTIs.

Many bacterial pathogens, including *uropathogenic E. coli* (**UPEC**), *Klebsiella pneumoniae*, *Enterococcus* species, *Streptococcus agalactiae, Staphylococcus aureus,* other *Staphylococci,* and *Proteus mirabilis* are significant clinical causes of UTI. Among otherwise healthy women aged 18-35 years, over 80% of UTIs are caused by UPEC and constitute a significant source of morbidity with more than 6 billion dollars in health care costs a year (1, 8, 62). In a recent prospective study on acute UTIs in young women, Hooten et al. found that the incidence was 50%-70% per year. With such high incident rates, UTIs also can recur with the risk of a second episode happening within six months was 24% in those with a history of one or more UTIs. The risk of a second within one year was 70%, making this the most frequently experienced bacterial infection among women (1, 8).

Urinary tract infections can be further classified into two clinical categories known as uncomplicated and complicated UTIs. An uncomplicated or acute UTI is often defined as cystitis occurring in the lower urinary tract (LUT), where the infection is restricted to the bladder. LUT infections usually occur in women with no known functional abnormalities to the urinary tract, who are not pregnant or have not had previous instrumentation performed, such as catheter placement. Uncomplicated infections usually do not result in long-term complications or renal damage (1, 8). A complicated UTI, or one which can occur in the upper urinary tract (UUT), has been described by Lee et al. as connected with anatomical or functional differences in the anatomy of a patient's urinary tract. Traumas to the pelvic floor and pelvic organ prolapse are examples of functional abnormalities seen in women with complicated UTIs (63). Functional abnormalities can disable the host's ability to respond appropriately to therapy and may cause tissue injury with potentially long-lasting effects. This condition is seen in individuals as pyelonephritis, or

inflammation of the kidneys, which can become severe and life-threatening septicemia if left untreated.

Although UUT infections are less common than LUT infections, there are various reasons why women are more likely to experience a urinary tract infection. An individual's susceptibility to UTI is complex and will vary greatly depending on genetic, biological and behavioral factors (64). Risk factors can range from the overuse of antibiotics (which can lead to antibiotic resistance, especially during repeated treatment), use of spermicides, sexual intercourse, pregnancy, female anatomy or genetics. In young women, spermicide use and frequency of sexual intercourse are the main risk factors evidenced by increased urethral and vaginal colonization (60, 65). In contrast, older women's predisposing risk factors are a high volume of residual urine, atrophic vaginitis, menopause and cystocele (60, 66).

Even though there are many risk factors contributing to the cause of a UTI, the majority of women who become infected experience an inflammatory process, where symptoms often include dysuria, severe abdominal pain, and even a fever with chills. Depending on which bacterial species predominates within the urinary tract at the time of infection, the pathophysiology is intricate and often determined by the specific adhesive virulent factors the etiologic agent possesses (64). A UTI will typically start with contamination of the periurethral area by a gut microorganism through an ascending mechanism. The vagina is a key anatomical site in the pathogenesis of UTIs, serving as a potential reservoir for uropathogens origination from the gut microbiome (56). The persistence of gut microbes within the urinary tract requires bacterial adherence via pili (fimbriae) or flagella, thus aiding in attachment to the bladder uroepithelium (30). Fimbriae were first described in the 1960s by J P Duguid, a professor at the University of Dundee. He described them as typically being associated with gram-negative bacteria such *E. coli and Salmonella spp*. They originated in

the cell's cytoplasm and project through the cell membrane and the cell wall (67). Type 1 and Type P fimbriae are the most important and commonly expressed virulence factors. Type 1 fimbriae are produced by 80% of UPEC species and play a critical role in bacterial adhesion and invasion of the urogenital tract (67).

Once a bacterial species, such as UPEC, attach and invade the uroepithelial cells, it will begin to colonize. This process triggers superficial cells to exfoliate and begin the cascade of inflammatory cells, such as neutrophils, to enter the bladder and help clear the extracellular bacteria. Although exfoliation is a host defense mechanism, many bacterial species can remain and evade the clearance by the immune system. The UPEC that are not cleared can create a biofilm. A biofilm is a microbiologically derived sessile community characterized by cells that are irreversibly attached to an interface or each other and embedded in a matrix of extracellular polymeric substances that they have produced (104). The communities of bacterial cells are known as intracellular bacterial communities (IBC). Production of polysaccharides in biofilm forming strains, such as UPEC, can facilitate aggregation, adherence, and surface tolerance, allowing better surface colonization (105). This will then allow the virulent bacterial strain to protect itself from immune responses like neutrophils, antimicrobial agents and stressors (30). The bacteria which form the biofilm produce toxins, that cause host damage to the epithelial cells and allow for the UPEC to replicate and enter the ureters. After colonizing the ureters, UPEC retrogrades toward the kidneys. Upon entry into the kidney parenchyma, bacteria such as UPEC can eventually enter the bloodstream and cause sepsis and bacteriuria. The ability of UPEC and other bacterial species to evade infection brings to light the fact that bacteria are ever-evolving to maintain their virulence (67, 68).

Treatment of a urinary tract infection has typically relied on antibiotics, but many researchers and clinicians continue to wonder if there are other effective antibiotic-sparing therapeutic approaches to manage UTIs. Antibiotic use for extended periods or repeated use carries a high risk of developing antibiotic resistance. One of the most common therapies is trimethoprim-sulfamethoxazole (TMP-SMX). The gold standard for symptomatic treatment of uncomplicated acute cystitis is a three-day treatment with TMP-SMX, with a percentage of eradication rate of 90% (63). The resolution of infections is defined as the clearance of culturable bacteria from the urine. Despite resolutions of acute infections, the rate of relapse or recurrence of UTI is as high as 15% within six weeks after the cessation of treatment (69, 70).

Prophylactic antibiotic therapy is commonly used for the management of recurrent UTIs. However, when antibiotics are discontinued, the infections tend to recur in these patients (69, 71). In a study of women with acute uncomplicated pyelonephritis, Talan et al. demonstrated that patients infected with TMP-SMX–resistant strains of bacteria and treated with TMP-SMX achieved only a 50% cure, compared with a 90% cure for those who received TMP-SMX for TMP-SMX–sensitive strains. With acute uncomplicated cystitis, where high urinary concentrations of TMP-SMX in the bladder urine might be expected to overcome in vitro resistance, more data are needed to ascertain whether resistance to TMP-SMX does indeed predict a higher rate of treatment failure (1, 72, 73).

Many additional treatments have also been proposed, including dietary factors such as cranberry juice, estrogen therapy, probiotics, and even vaccines. Probiotics have become a choice that has been used for women who commonly experience recurrent UTIs (RUTIs). RUTIs are defined as UTIs that occur at a frequency of two to three infections throughout the year. Probiotics (commonly called “good bacteria”) include members of the *Lactobacillus* species. Lactobacilli

produce antimicrobial compounds such as lactic acid, bacteriocins and hydrogen peroxide that are toxic to many microorganisms at vaginal concentrations (63). Women who experience RUTIs have been found to have a significant lack of *Lactobacillus* species within their vaginal microbiota and will more commonly have a mixture of different harmful strains of UPEC present.

Data from multiple clinical studies suggest that a woman's vaginal microbiota affects her susceptibility to UTIs. For example, it has been shown that women with bacterial vaginosis have a higher UTI risk than women with lactobacilli-dominated vaginal microbiota (56, 57, 74, 75, 76). Oral and intravaginal probiotic agents have been used to try and help prevent RUTIs. Although oral lactobacillus products have proven unsuccessful, intravaginal products have shown success. Reid et al. showed a 43% reduction in the incidence of UTI in women with a history of recurrent UTI by using hydrogen peroxide-producing 108-9 *Lactobacillus acidophilus* (Vivag®, Denmark) twice daily for six days. The lactobacilli were delivered in a tampon vaginally (67). An additional pilot study carried out utilizing suppositories containing *Lactobacillus crispatus* GAI 98332 demonstrated a significant reduction of RUTI after a 12-month treatment course (63, 77). The addition of probiotics has proven helpful for many women, even though the reasons why women with an increased diversity of their vaginal microbiome are more susceptible remain unclear. Additional randomized, double-blind, placebo-controlled studies of adequate sample size using a carefully selected probiotic strain are further needed to ascertain whether this approach is truly effective or just case-dependent (56).

Additionally, vaccines preventing UTIs have been encouraged for further consideration and review for the most susceptible women. As adherence has a crucial role at nearly every step of UTI pathogenesis, one attractive strategy is the development of anti-virulence therapies (30). Immunization with UPEC antigens can stimulate a mucosal immune response that may effectively

prevent experimental UTIs, and increases in urinary and serum antibody titers correlate with reductions in bladder bacterial load and infection duration (30). These data provide encouragement that an effective UPEC vaccine can be developed (78). Specific targets are used to create the mucosal immunity seen in healthy women. One such target is via Fim H adhesion of type 1 fimbriae. Electron microscopy data suggest that these bacterial cell wall projections bind to the urothelial mannosylated glycoproteins uroplakin Ia and Ib via the adhesin subunit FimH, located at the fimbrial tip (67). Intravesical inoculation of old world primates with Fim H protected them from subsequent experimental UTI (79). Similarly, inoculation of P fimbriae adhesion PapG (Pap DG vaccine) has been shown to protect against RUTI in a nonhuman primate model (80). The UPEC adhesin, PapG, which is the tip adhesin on P fimbriae, binds to Gal(α1-4)Gal-specific glycosphingolipids on kidney epithelium (P blood group antigen) and likely plays an important role in UPEC human kidney colonization (78).

Although type 1 and P fimbriae have elicited an immune response for protection against adhesion, most notably via the Pap DG vaccine, their use is often combined with other subunits to create an even stronger correlation. One additional type of fimbriae that have been notably studied, are known as Dr fimbriae. Dr fimbriae are a family of adhesins and a commonly encountered virulence factor associated with many strains of E coli (67). Dr fimbriae can also bind to the uroepithelium and can cause pathogenesis of UPEC, causing further destruction to the urinary tract. Mouse UTI models using Dr positive UPEC strains maintained higher bacterial loads than mice infected with a Dr fimbriae knockout. Vaccinating mice with purified Dr fimbriae produced high titers of serum anti-Dr antibodies and significantly reduced experimental UTI-associated mortality but did not affect the rate of bladder or renal colonization (78, 101). Further studies are needed to thoroughly test the potential of Dr fimbria, P fimbriae, and type 1 fimbriae as UPEC

vaccine targets to improve vaccine efficacy. The substantial diversity that exists within classes of UPEC virulence factors, such that not every UPEC strain expresses the same set of virulence-associated genes during infection, is proving to be a challenge to the development of a one-vaccination approach protecting against all diverse strains of UPEC, as well as other common non-UPEC UTI (78,81). Although the genomes of pathogenic *E. coli* frequently encode many more virulence factors than commensal *E. coli* strains, the absence of a required core set of virulence factors tremendously complicates UTI vaccine design and development (78, 82, 83).

Lastly, estrogen has also been pursued as a treatment option for women who experience RUTIs, particularly post-menopausal women. In general, women who are experiencing menopause will have significantly lower estrogen levels which are thought to predispose them to UTIs. The absence of estrogen decreases the volume of vaginal muscles causing secondary slackness of the ligaments holding the uterus, the pelvic floor, and the bladder, thus resulting in prolapse of the internal genitalia (66). In addition, the menopause-associated estrogen deficiency can also cause atrophic changes within the urogenital tract that are usually associated with urinary symptoms, such as frequency, urgency, nocturia, urinary incontinence and RUTI (63). The presence of estrogen can help stimulate the proliferation of lactobacillus in the vaginal epithelium, reduce pH, and prevents vaginal colonization of uropathogens. However, some individuals do not respond to vaginal estrogen replacement therapy. In a study by Sobel et al., estradiol released from the vaginal pessary did achieve increased maturation of vaginal epithelial cells. In contrast with previous studies, this form of estrogen administration failed to achieve its functional goal because the pH remained abnormally high (pH was 5.3), and a lactobacillus dominant vaginal flora was not achieved (102). Although we know that estrogen has been associated with positive clinical

effects, there is still much work to be done in regard to refining this treatment regimen to make it broadly applicable for post-menopausal women.

Continuing Research on UTIs

UTIs have long been studied, and even though there are treatment options available like those previously discussed, there are still many aspects of UTIs that are still unknown, making it continually challenging to study. One of the areas of UTI research that has still not been perfected is defining a definitive diagnosis. For many decades researchers have used the same diagnostic technique, the urine culture. Although urine cultures continue to be the gold standard, this process can still take a considerable time to receive results. Often clinicians will begin treatment for UTIs prior to getting the results back from the culture. Without knowing what bacteria or group of bacterial agents is invading or what antibiotic it is susceptible to, empirical antibiotic treatment risks increasing antibiotic resistance. Patient care could benefit greatly from the development of a rapid (i.e., patient bedside), accurate, inexpensive test that would allow the practitioner to decide whom to treat at the time of examination (1).

Additionally, there have been no definitive answers for a cure. Although there are many treatment options, there does not seem to be one that affects all women with the same efficacy. Developing a preventative measure and a cure for the problem to help women stricken with the recurrence of UTIs has and continues to be something researchers and clinicians must continue to piece together. Using several animal models, we can use the knowledge we have to study those unanswered questions, bringing us one step closer to helping those who continually struggle with urinary tract infections.

Animal Models used in UTI Research

Biomedical research has allowed scientists to uncover discoveries for new treatment options, devices, and rapid tests that allow clinicians to make a timely diagnosis. Through the humane and proper use of animals, biomedical research can help us find solutions to significant public health problems that continue to plague humans and animals. Animal models generate invaluable information that can be extrapolated to benefit human and animal health. The earliest mention of establishing infection in animal bladders was in 1873, when Fels and Ritter inoculated canine bladders to induce cystitis using urethral ligation (84). Larger animal models, such as rats and rabbits, continued to be important for UTI research, until the first murine model of UTI was described in 1967 as an experimental infection model for pyelonephritis (85).

The number of animal models for urinary tract infections is vast, and many have not been completely explored. These include rabbits, rats, dogs, cats and zebrafish. However, three animal species extensively used for UTI research have given us an abundant amount of information that could help lead to an overall cure, as well as better diagnostic testing. The models that are most commonly used and will be further discussed are swine (*Sus scrofa domestica*), non-human primates (*Macaca spp*), and, most notably, mice (*Mus musculus*). As the ability to understand and broaden our knowledge of the UTI increases, the capacity of UPEC to create such a diverse niche in many different species has prompted researchers in the development of many animal systems to appreciate the virulence potential, adaptability, and evolution of these unique and ubiquitous pathogens (19).

Swine as a UTI model

Swine have been used as an animal model for over 30 years, particularly in areas such as xenotransplantation and cardiology. However, there are many advantages of utilizing swine as a UTI model to simulate the pathogenesis, immune function, and treatment options for women most commonly affected with UPEC. Given that UTI remains a considerable challenge in hospitals and the community, the considerable fundamental biological investigation conducted over the past decades must be translated into practical, novel treatment regimens. Emerging evidence suggests that the swine anatomy, their overall size, and the homology of swine immune system proteins to humans are closer than human and mouse homologs (19, 86, 87, 88). This data supports the use of pigs as the transitional species between mice and humans to help model immune functions relevant to humans (89).

Experimentally-induced UTI models have largely been created using transurethral catheterization, which has also been highly utilized in the mouse model. The catheterization process allows the researcher to instill bacteria directly into the bladder through the urethra of the female by catheterization. This mode of infection can ensure the ascension of bacteria from the bladder to the UUT for evaluation and, additionally, retention of bacteria in the bladder to study LUT infections. In a study done by Nielsen et al., they evaluated the use of UPEC strain UTI89, 12-, 16- and 23-days post-infection in female swine (89). Their analysis revealed that 24 hours post-infection (hpi), strain UTI89 is present in the urine in significant numbers averaging 10^7–10^8 CFU/ml. Analysis of the urine specimens collected at later time points revealed persistent bacteriuria (>10^5) throughout experiments lasting up to 23 days, indicating the establishment of a chronic infection. In response to bacterial inoculation, the pigs also exhibited an inflammatory response of fever, bladder mucosal edema, and elevated granulocytes, all characteristics of UTI in

the human host (90, 91, 92). In comparison to humans and pigs, mice are highly resistant to endotoxin shock and respond with hypothermia rather than hyperthermia (86). This is a limiting factor for using mice as models for studying moderate to severe infections associated with hyperthermia, making swine a more parallel animal model to study human disease.

Staerk et al. have evaluated if virulence factors such as type 1 fimbriae (T1F) are essential for low dose inoculation of UPEC UTI89 in female swine to create a model for cystitis. Type 1 fimbriae are the primary virulence factor that allows for the attachment of *E. coli* to the bladder wall for persistent infection within the urinary and genital tract in humans. Using a type 1 fimbriae deficient mutant of UTI89 compared with UTI89 wild type strain, this group concluded that type 1 fimbriae deficient mutant strain of UTI89 only colonized the bladders in 16.6 % (1 of 6) of pigs. This probability of infection was significantly smaller compared to the wild-type strain, which successfully infected 100 % (6 of 6) of pigs. These results have provided new insight into the infectious potential of UPEC by showing that low infectious doses are sufficient for successful infection in pigs. Type 1 fimbriae are a key mediator of inflammation and add to the support that adhesin-receptor interactions are a primary signaling route for the immune response and may function as an adjuvant to stimulate an immune response against other antigens.

The possibilities for swine to become a better model for UTIs are increasing from the information already uncovered. There continue to be significant limitations, which can include their size, a need for personnel to have specialized handling skills as well as larger facilities that could increase the cost of retention, and a lack of genetic tools to generate transgenic pigs readily. However, their use continues to benefit research efforts to assess novel therapeutics in preclinical trials prior to human studies (19).

Non-Human Primate as a UTI model

Non-human primates (NHP) of many different species and origins have also been thoroughly studied in many aspects of biomedical research. Their use as a primary research species has been applied to pre-clinical data to study different vaccine candidates against SARS-CoV2, measles, and human papilloma virus. In addition, they continue to be models for HIV/SIV, tuberculosis, and the Ebola virus, which have truly shaped our approach to epidemiologic screening of diseases, prevention and treatment regimens. Due to the closer genetic relationship, monkey models for UTI may be more relevant for understanding the human disease and for the future design and testing of new therapeutic approaches (93, 94).

Compared with other animal models, UTI inoculation techniques used to instill different strains of *E. coli* in nonhuman primates commonly utilize catheterization of only the urethra. Non-human primate UTI models have also been performed through the inoculation by a catheter placed in the vagina, emulating an ascending infection as seen in human women when contaminated with UPEC from gut microorganisms. UPEC species that originate in the gut continuously use their adherence factors to bind to the bladder wall to cause infection. UPEC can express type 1 and p fimbriae when colonizing NHPs, therefore NHPs have been used to study these specific UTIs.

A study by Roberts et al. used both vaginal and urethral inoculation techniques of UPEC strains DS17 and 1103. Twenty total female *Cercopithecus aethiops* were put into two study groups. The first group contained 5 African vervets that were inoculated vaginally with strain DS17 and 5 were inoculated vaginally with strain 1103. The second study group contained 5 African vervets inoculated urethrally with strain DS17 and 5 with strain 1103 respectively. This study showed that colonization of the vagina was favored by the presence of either type 1 fimbriae alone or by adherence of bacteria with both P and type 1 fimbriae, and yet colonization was not

always associated with invasive disease. Using the BALB/c mouse model compared to the NHP, Schaeffer et al. used wild-type *E. coli* strains capable of producing different levels of type 1 fimbriae (95, 96). The importance of the degree of fimbriae production (piliation status) was shown; when piliation increased, so did the incidence of colonization. Their studies suggested that type 1 fimbriation is important for colonization since strains containing only P fimbriae were less effective in colonizing the urinary tract. However, renal colonization was greatest when the *E. coli* contained both P and type 1 fimbriae.

In addition to commonly evaluated adherence factors, non-human primates have been used to study the effects of antibiotics and antibiotic resistance after inoculation with different strains of *E.coli*, particularly strain DS17. In a study done by Winberg et al., the authors evaluated the genital flora once colonized with *E. coli* and determined if resistance occurred after infection and if that resistance could be restored through other methods such as the introduction of *Lactobacillus spp.* They found that when the P-fimbriated, UPEC strain DS17 was instilled into the vagina on one occasion, persistent vaginal colonization occurred in only 10 of 58 *Macaca fasicularis* evaluated. Thus, the vagina could protect itself and resist colonization in 83% of the experiments (93). When a daily intravaginal flush with amoxicillin followed the *E. coli* administration, after six administrations, the colonization resistance broke down, and persistent vaginal colonization with *E. coli* DS17 was observed in all 16 cynomolgus monkeys investigated in this study group. The effect of intravaginal administration of nitrofurantoin and trimethoprim on vaginal colonization resistance was also studied (93). Neither drug promoted vaginal *E. coli* DS17 colonization compared with control experiments. Finally, to examine whether there was a cause-and-effect relationship between the partial elimination of the normal vaginal flora and the increase in UPEC colonization, they studied the effect of implanting either *Lactobacilli* or total indigenous

vaginal flora from healthy primates. In contrast, fresh vaginal fluid obtained by swabbing the vagina of a healthy, mature monkey and then directly transferring the vaginal fluid to the recipient monkey (without culturing) eliminated the vaginal *E. coli* in all four cynomolgus monkeys evaluated.

The NHP model, particularly vervet monkeys (*Chlorocebus aethiops*) has additionally been used to examine the ability to mobilize copper (Cu) to the urine during UTI. Copper has been established as a protective host factor in the urine of people experiencing UTIs that limits bacterial growth in the urogenital tract. Hyre et al. hypothesized that copper is mobilized to the urine during experimental UTI in NHPs, similar to observations from humans with clinically diagnosed UTI. This study found that although copper was present in larger amounts in mouse urine than in human or NHP urine, it did not continue to mobilize or accumulate once a UTI was induced experimentally with UPEC. However, unlike the mouse model, the NHP model did demonstrate that all four adult females inoculated with UPEC strain CFT073 by cystocentesis readily colonized the urinary bladder, and urinary copper levels were significantly higher during UTI than pre-UTI urine samples. These findings confirmed that copper is mobilized to the urine as part of the innate immune response activated during UTI in the NHP model. The similarities between NHPs and humans continue to illustrate their ability to serve as a more relevant model for human UTI, compared to mice.

Much work remains to be done. For example, the mechanisms through which *E. coli* establishes itself on the external genitalia and the mechanisms through which the resident flora excludes an intruder must be better understood (97, 98). However, UTI research can greatly benefit from being conducted in NHP models because of their substantial anatomic and immunologic similarities with humans. Currently, the literature describing NHPs as a UTI model is still very

limited, but their use is valid, and scientists should strongly consider their continued use to better understand UTIs and the mechanisms associated with the instillation, colonization and infection of humans (99).

Mouse as a UTI model

The murine model continues to be the most commonly used species in biomedical research today, with its use constituting over 98% of published research. Mice are commonly used in studies involving gnotobiotics, toxicology, cancer, and most notably, urinary tract infections. Although the first murine model for pyelonephritis was developed in 1967, the first murine model for human UTI research was described in 1975 by Kalmanson et al., and since then, refinements have been implemented to address critical questions germane to the pathogenesis, prevention, diagnosis and treatment of UTI.

Mice hold considerable similarity to humans when studying the effects of urogenital and renal infections with UPEC. Rodents have common immunological factors and similar anatomical features within the urinary tract (18, 19). Mice, in particular, have been a very good model for systemic complications, mimicking human disease by developing bacteremia and even sepsis after experimental UTI. The mouse model also offers a diverse range of genetic variability, an extensive collection of commercially available transgenic lines, and the ability to generate new transgenic lines in a reasonable time frame allowing researchers to test specific host factors and immune responses in transgenic and knockout mutants (19).

Despite their commonalities with humans, mice do not naturally develop UTI and at this time the field is still unsure of why this is (89). Therefore, to study UTIs in mice, bladder instillation techniques were developed. Hagberg et al. described the creation of an ascending,

unobstructed UTI in female CBA mice instilled with *E. coli* in the bladder via a transurethral catheter (16). This model has since been adapted for other uropathogens such as *Proteus mirabilis* and *Enterococcus faecalis* (Jones et al., 1990; Shankar et al., 2001). The most common mode of infection is inoculation by transurethral (intra-bladder, intravesical) delivery of bacteria directly into the bladder following insertion of a small catheter through the urethral orifice of female mice (18). This instillation method helped replicate the attachment of human-derived UTI isolates to murine urothelial cells, which was considered essential for understanding human infection. This method requires catheterizing the urethra for bladder colonization; thus, the factors that determine vaginal and periurethral colonization in humans, and bacterial entry from the periurethral area into the bladder, are bypassed by direct instillation of bacteria into the urinary tract in murine models (18). The importance of this disconnect with murine UTI models remains unknown, leaving many unanswered questions regarding if mice can accurately emulate events occurring at the interface of the human host and UPEC.

Humans and mice also differ in some innate immune system parameters. During UTI, the host innate immune system's primary responsibility is to recognize the presence of pathogens such as UPEC through the use of pathogen recognition receptors (PRRs). The main group of PRRs that allow pathogen recognition is known as toll-like receptors (TLRs). TLRs are expressed in both humans and mice; however, not all TLRs possess the same properties within these two species. For example, TLR10 is found only in humans and TLR11-13 are found only in mice (100). TLR4 is the most well-defined of the 13 TLRs when studying UTIs in both mice and humans; however, each expresses different levels which can initiate the signaling process to begin the innate immune response. This could also lead to different responses between mice and humans, creating a

translational knowledge gap and causing erroneous conclusions regarding viable treatment options for women. Further exploration for a definitive understanding of both species is necessary.

Although mice models have their limitations, we continue to find new information that has the potential to understand further how we can replicate the human pathogenesis for continued study. Much of the research involving mouse UTIs revolves around the fact that many women who experience infections are post-menopausal with a limited amount of endogenous estrogen, making them more susceptible to disease. Menopause has been experimentally implemented in many ways using mice models. One way is through the use of ovariectomized (OVX) mice (i.e., surgical removal of both ovaries), which produce negligible circulating estrogen levels, mimicking menopausal/post-menopausal women.

In a study completed by Wang et al., they were successfully able to relay that estrogen-deficient or OVX mice have severe pro-inflammatory responses where IL6 and inflammatory cells such as neutrophils are recruited to the site of infection after UPEC inoculation (37). They observed significantly higher levels of IL-6 in sera from infected OVX mice than from control mice at 6 hpi, suggesting an overall enhanced systemic and luminal pro-inflammatory response to infection in OVX mice. This research effort was also able to determine if estrogen replacement therapy would help to reverse or help restore the bladder and vaginal environments to their pre-menopausal state. Although exogenous estrogen (E2) administration did not lower or impact the bacterial loads in the urine, there were significantly lower IL-6 serum levels and low levels of inflammation, based on histology results, in the bladder mucosa in OVX mice treated with estrogen relative to untreated OVX mice. Lastly, they concluded that bladders from OVX mice that received E2 supplementation contained fewer biofilms than mice that were ovariectomized but did not receive E2. At 14 dpi, OVX mice receiving E2 before infection harbored similar numbers of biofilms as control mice,

indicating that the enhanced biofilm formation observed in OVX mice was primarily due to estrogen deficiency (37).

In addition to the OVX model, researchers have used exogenous estrogen that will keep mice in the estrus phase of their estrous cycle to investigate the impact of estrogen on bacterial colonization within the urogenital system. Studies have shown that estrogenized mice are more susceptible to infection and urinary tract colonization. Estrus is characterized by the presence of abundant cornified epithelial cells and an absence of leukocytes in the vaginal lumen (43), conditions that may favor *E. coli* colonization. O'Brien et al., examined these effects in estrogenized mice and how they respond to UPEC instillation. Even though estrogenized mice showed an apparent lack of vaginal inflammation at 12 dpi, despite having sustained, robust *E. coli* colonization, this may be attributable to local immunosuppression due to estrogenization, implying a protective role from further systemic or local infection.

Estrogenizing mice by supplementation of β-estradiol 17-valerate (E2) has been very beneficial in understanding the role of estrogen in modulating UTIs in the mouse model. However, up until this point, no study has explored the impact of endogenous changes in estrogen levels during an estrous cycle on the onset, progression, and severity of UTIs. In the model created here at Texas A&M I sought to explore if mice experiencing estrus without E2 supplementation are susceptible to high levels of colonization and systemic dissemination of UPEC, compared to those mice in other stages of the estrous cycle. This refined UTI model also eliminates the requirement for urethral catheterization and replaces it with intravaginal inoculation of bacteria to replicate a model of ascending UPEC similar to that of human UTIs.

CHAPTER II

Vaginal Inoculation of *Uropathogenic E. coli* During Estrus Leads to Genital and Renal Colonization

ABSTRACT

Urinary tract infection (UTI) is one of the most prevalent bacterial infections, particularly in women, children, and the elderly. Uropathogenic E. coli (UPEC) is the predominant etiological agent of UTI. Uropathogens are directly instilled in the urinary bladder, bypassing lower urogenital tract, in the widely-used murine model of UTI. We assessed whether vaginal inoculation of UPEC led to UTI, and how stages of the estrous cycle would impact bacterial colonization in mice. Mice in proestrus, estrus, metestrus, and diestrus were identified by vaginal cytology, and inoculated with UPEC in the vaginal tract. Mice were euthanized 1-day post infection, and bacterial loads in the urogenital tract, liver, and spleen were enumerated. Mice in estrus exhibited the highest and consistent UPEC burden in all organs, except the bladder. Vaginal inoculation resulted in bladder colonization in a UPEC strain-specific manner. In contrast, transurethral inoculation of UPEC led to bladder colonization. Importantly, inoculation by both routes led to vaginal and uterine colonization, and concomitant systemic dissemination to spleen and liver. Kinetics of bacterial colonization over 2 weeks following vaginal inoculation was comparable in the urogenital tract. Tissue sections revealed the induction of vaginitis and cystitis upon vaginal instillation of UPEC. In summary, vaginal inoculation of UPEC in mice during estrus represents a novel approach to investigate infection of the kidneys and genital tract, and systemic dissemination from the urogenital tract. Our findings suggest that estrogen primes the urogenital tract to create a conducive milieu for UPEC colonization.

INTRODUCTION

Bacterial infection of the urinary bladder is a ubiquitous infectious condition (1). Urinary tract infection (UTI) results in ;11 million physician office visits, 1.7 million emergency room visits, and 470,000 hospitalizations, with an annual direct cost of approximately $3.5 billion in the United States (2–5). Cystitis, inflammation caused by infection of the urinary bladder, is the most common clinical presentation of UTI (6). Less common but more serious outcomes of UTI include kidney infection (pyelonephritis), bacteremia, and sepsis (6, 7). Women are at a 4-times-higher risk of developing UTI than men due to anatomic differences in the urogenital tract (8). Factors that increase the risk for UTI include diabetes mellitus, age (children and the elderly), catheter use, anatomic/physiological abnormalities of the urinary tract, or urolithiasis (7, 9, 10). Recurrent UTI is also common among high-risk groups. Uropathogenic Escherichia coli (UPEC) is the predominant etiological agent of UTI (7, 9, 11). Uropathogens are found in the human gut microbiome (12,13) and infect the urinary bladder by colonizing the lower urogenital tract followed by ascension along the urethra (14).

Mouse models are used to elucidate the pathogen and host factors that determine the outcome of UTI (15–19). Murine models currently used to investigate experimental UTI rely on the intravesical instillation of uropathogens through a transurethral catheter. Colonization of the urinary bladder with uropathogens results in cystitis, inflammation of the urinary bladder. Uropathogens often cause ascending infections leading to pyelonephritis and systemic dissemination from the urinary tract. UPEC and other uropathogens are known to establish an intracellular niche within the urothelium that lines the bladder lumen (20–23). Recently, UPEC has been demonstrated to invade and form intracellular reservoirs in vaginal epithelial cells, reminiscent of the phenomenon observed in the urothelium (24). Sites of inoculation of

uropathogens in the existing murine models of UTI bypass lower urogenital tract colonization to directly introduce uropathogens in the urinary bladder. A better understanding of the mechanisms involved in uropathogen colonization in the lower urogenital tract and their impact on the development of UTI is critical for developing novel intervention strategies against this important public health problem.

Here, we sought to evaluate the impact of vaginal inoculation of UPEC on colonization of the urogenital tract in female CBA/J mice. We also assessed which stage of the estrous cycle would be more permissive to UPEC colonization. Our results reveal that vaginal instillation of UPEC led to consistent genital and renal colonization for up to 2 weeks. However, vaginal instillation results in poor bladder colonization, compared to intravesical instillation of UPEC. Furthermore, a UPEC strain-dependent effect on bladder colonization was also evident after intravaginal inoculation. In summary, we present a novel approach to investigate urogenital colonization following inoculation of UPEC in the vaginal tract.

MATERIALS AND METHODS

Mice. Experiments were conducted in accordance with the Animal Welfare Act, and all protocols were approved by the Institutional Animal Care and Use Committee of Texas A&M University (IACUC-2018-0362). CBA/J female mice (6 to 8 weeks old; JAX Laboratories) were housed under specific-pathogen-free conditions in an AAALAC-accredited biosafety level 2 (BSL2) animal facility at Texas A&M University. Mice were assessed for pathogen status, by surveillance testing using dirty bedding sentinels and exhaust air dust, for the following agents: lymphocytic choriomeningitis virus, mouse adenovirus, *Mycoplasma pulmonis*, Theiler murine encephalomyelitis virus, pneumonia virus of mice, reovirus, Sendai virus, mouse hepatitis virus, minute virus of mice, mouse parvovirus, mouse rotavirus, ectromelia virus, polyomavirus, pinworms, and fur mites. All mice were negative for the above-mentioned pathogens for the duration of the study.

Vaginal cytology. Mice were acclimated to our housing facility for at least 2 days before the collection of samples. Mice were restrained by scruffing and immobilizing the base of the tail for the collection of vaginal lavage fluid with 50 mL of sterile deionized water dispensed from a micropipette. Smears of vaginal lavage fluid were prepared, stained with DiffQuick (Fisher Scientific), and evaluated by light microscopy. Stages of the estrous cycle were determined based on the presence and abundance of nucleated epithelial cells, cornified epithelial cells, and/or leukocytes, as described previously (42, 43). Vaginal cytology was performed for 4 consecutive days, followed by a rest phase of a week, and repeated prior to inoculation of UPEC. A rest phase was included to avoid accidental induction of anestrus.

Bacterial strains and growth conditions. Clinical UPEC strains CFT073 and UTI89 (49, 50) and CFT073 mutants with the type 1 pilus promoter locked in the on or off orientation (58)

were cultured in LB broth or agar (tryptone at 10 g/L, yeast extract at 5 g/L, NaCl at 5 g/L, and agar at 15 g/L). Cultures were incubated at 37°C and aerated by shaking at 200 rpm. UPEC strains were cultured to stationary phase in LB broth, washed, and resuspended in PBS prior to inoculation in mice.

Hemagglutination assays. Hemagglutination titers were determined using guinea pig and human erythrocytes to assess the presence of type 1 and P pili, respectively (25, 26). The inoculum, prepared as described above, was serially diluted 2-fold in PBS and mixed with an equal volume of a 3% (vol/vol) suspension of erythrocytes (Innovative Research). Strains CFT073 fim-ON and fim-OFF were used as positive and negative controls for type 1 pilus-dependent hemagglutination. The mannose dependence of hemagglutination was validated with a-methyl D-mannopyranoside (10 mg/mL; Sigma). Plates were incubated at room temperature for 30 min. The reciprocal of the fold dilution of bacteria in the last well with agglutination was recorded as the hemagglutination titer. The experiment was repeated independently, and results were analyzed by a t test.

Vaginal instillation of UPEC. Mice (n = 10/group/time point) were instilled with approximately 2 CFU/mouseofUPECstrainCFT073orUTI89in50mL of PBS in the vaginal tract. Urine and vaginal lavage fluid were collected prior to euthanasia (1, 7, or 14 days after inoculation). After euthanasia, blood, vagina, uterus, bladder, kidneys, spleen, and liver were collected aseptically, homogenized in PBS, and cultured on LB agar. After 1 day of incubation, viable bacterial counts were determined to assess genital, urinary tract, and systemic colonization. 108 Cohousing after vaginal UPEC instillation. To rule out contamination by UPEC from bedding, we performed cohousing experiments with UPEC-instilled, PBS-instilled, and naive mice. Mice in all groups were in estrus during UPEC/PBS instillation. Mice were vaginally inoculated with UPEC strain CFT073 or UTI89 and cohoused with control mice in a cage (one UPEC strain/cage)

with autoclaved bedding for 1 day. Samples were collected and processed at 1 day post-inoculation, as described above.

Gentamicin protection assay to determine intracellular UPEC loads. In an independent experiment, vaginas from mice in estrus that were infected with UPEC strain CFT073 or UTI89 intravaginally were collected at 1 day post-inoculation and bisected along the longitudinal axis. One half was homogenized to determine the total (extracellular and intracellular) UPEC load. A gentamicin protection assay was performed as reported previously by Brannon et al. (24). Briefly, the other half of the vagina was rinsed in PBS, treated with gentamicin (100 mg/mL) for 2 h, and extensively rinsed in PBS prior to homogenization to determine plate counts.

Intravesical instillation of UPEC. Mice were anesthetized with tribromoethanol, and the bladder was catheterized transurethrally. UPEC, comparable to the inoculum instilled in the vagina, was instilled in the bladder, as we and others have described previously (15, 27, 59). Sample collection and tissue processing were performed essentially as mentioned above for the vaginal UPEC instillation experiments.

Histopathology. The vagina, uterus, bladder, and kidneys (entire organs) from naive, PBS control, and UPEC-inoculated mice were fixed in formalin, embedded in paraffin, and sectioned. Hematoxylin and eosin (H&E)-stained sections were evaluated by a board-certified veterinary anatomic pathologist who was blind to study groups. Tissue damage scores were assigned based on the degree of inflammation, immune cell infiltration, integrity of the mucosa, the presence and nature of luminal contents, exfoliation of epithelial cells, tissue edema, the presence and extent of inflammatory exudate, micro abscess formation, the presence or absence of bacteria, and the presence or absence of tissue injury. Tissue scores were assessed on a scale of 0 (healthy) through 4 (extensive inflammation and damage).

ELISA. Urine and homogenates of the vagina, uterus, bladder, and kidneys were used to determine the abundances of myeloperoxidase (MPO), IL-1b,IL-6,andTNF-a, as we have recently described (28, 59). Urine samples were normalized based on the creatinine content, and tissue samples were normalized to the weight of organs. Optical density values were used for semiquantitative comparison of between-group changes in the analyte levels.

Statistical analysis. Experiments were repeated at least twice independently. Results were analyzed in Prism 7 (GraphPad) by a t test, a Mann-Whitney U test, or analysis of variance (ANOVA). A P value of , 0.05 was considered a statistically significant difference.

RESULTS

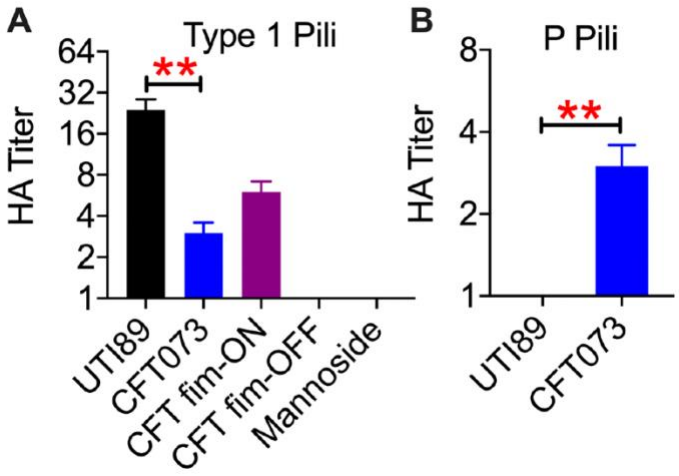

Fig. 1. Type 1 and P pili production in UPEC. Agglutination of guinea pig (A) and human (B) erythrocytes by clinical UPEC strains (UTI89 and CFT073) were evaluated. Mutants in strain CFT073 that constitutively produces type 1 pili (CFT fim-ON) and lacks type 1 pili (CFT fim-OFF) were used as positive and negative controls, respectively. Mannoside is an inhibitor of type 1 pili-mediated hemagglutination and was used as a negative control. Mean and SEM from independent experiments is presented. **P<0.01, t-test.

Vaginal instillation of UPEC during various stages of the estrous cycle. First, we assessed the effects of the stage of the estrous cycle on UPEC colonization in the urogenital tract of female CBA/J mice. Vaginal cytology was used to establish that these mice were cycling and to assess the stage of the estrous cycle at the time of bacterial instillation. UPEC strain CFT073 (;2 108 CFU) was instilled into the vaginal tract (n = 8 to 14 mice/stage of the estrous cycle). The presence of type 1 and P pili, well-characterized virulence factors of UPEC, was evaluated by agglutination of guinea pig and human erythrocytes, respectively (25, 26). UPEC strain CFT073 produced both type 1 and P pili but with a lower level of type 1 pili than strain UTI89 (Fig. 1A and B). This assay was validated with UPEC mutants and a chemical inhibitor (Fig. 1A). Strain UTI89 produced a

higher level of type 1 pili than CFT073 and did not produce type P pili (Fig. 1A and B). Mice were euthanized at 1 day post-inoculation, and bacterial loads in the urine, vaginal lavage fluid, blood, and organs (urogenital tract, spleen, and liver) were determined. We did not detect UPEC in blood samples by plate counts (limit of detection = 10 CFU/mL). Cytological evaluation of vaginal lavage fluid revealed the presence of nucleated epithelial cells during proestrus (Fig. 2A), cornified epithelial cells during estrus (Fig. 2B), a mixture of cornified cells and polymorphonuclear cells during metestrus (Fig. 2C), and all these cell types during diestrus (Fig. 2D). A few epithelial cells were detected during urine cytology, but they did not appear to harbor UPEC (see Fig. S1 in the supplemental material).

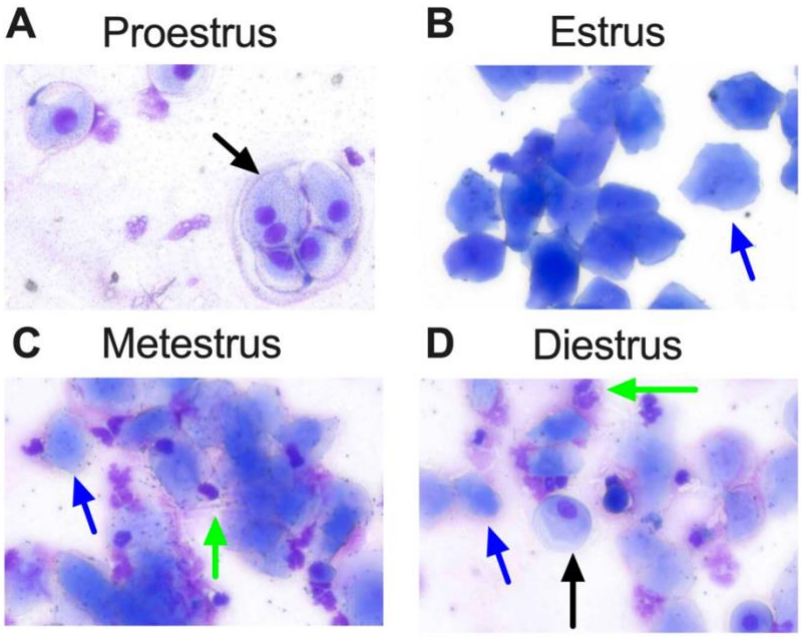

Fig. 2. Vaginal cytology reveals estrous cycle-associated changes. Mice were instilled with UPEC strain CFT073 in the vagina during various stages of the estrous cycle. Cytological evaluation of vaginal lavage was performed at one day post-inoculation. (A) Nucleated epithelial cells (black arrow) were observed during proestrus. (B) Estrus was characterized by the presence

of cornified epithelial cells (blue arrow). (C) Cornified and polymorphonuclear cells (green arrow) were evident during metestrus. (D) Diestrus was marked by the presence of all these cell types. A representative image for each stage of the estrous cycle at 400 X magnification is included here.

Mice in estrus and metestrus exhibit higher bacterial colonization. Mice in proestrus were poorly colonized by UPEC, with bacterial counts below the limit of detection (10 CFU) in many mice at all sites and samples that were evaluated (Fig. 3A to H). Bacterial colonization was higher in mice in other stages of the estrous cycle at all sites/samples that were evaluated (Fig. 3A to H).

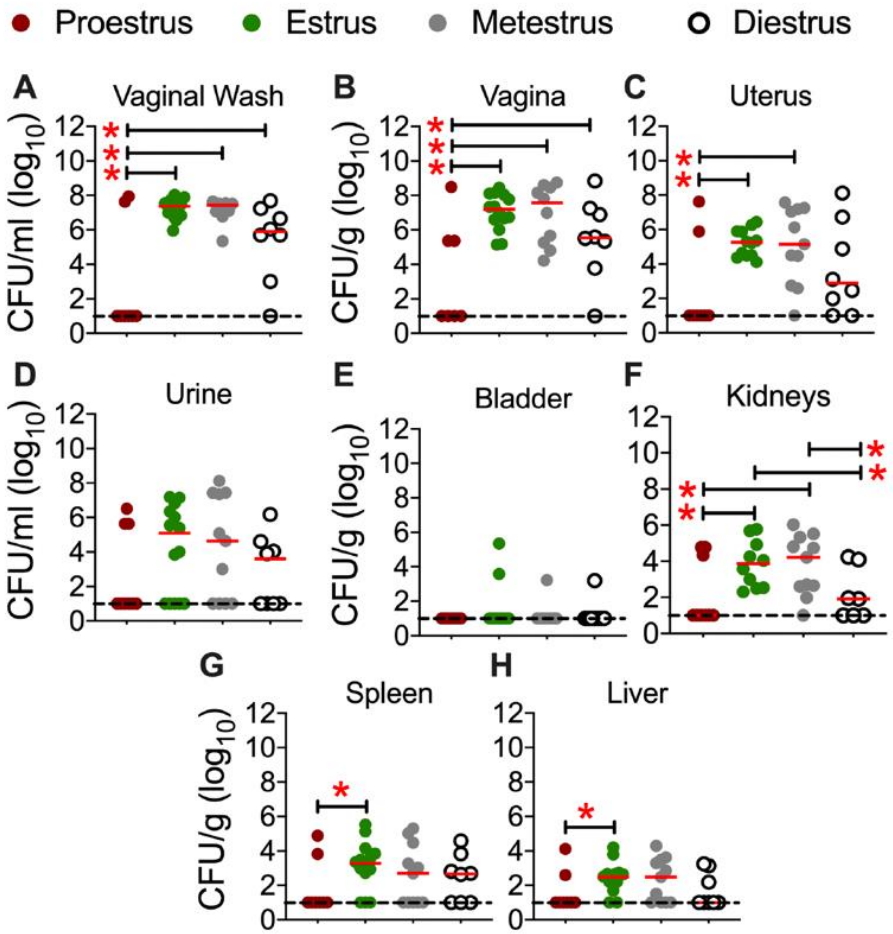

Fig. 3. UPEC colonization in the urogenital tract during various stages of the estrous cycle. Female CBA/J mice (N=8-14 mice/stage of the estrous cycle) were inoculated with UPEC strain CFT073 in the vaginal tract. Urine and vaginal lavage were collected one day post-inoculation,

prior to euthanasia. Bacterial load in the samples were determined by viable counts and normalized to volume/weight of samples. Each symbol corresponds to results from a mouse, and red bars indicate median. Data from the 613 two replicates are depicted here. Dotted line corresponds to the limit of detection (10 CFU). *P<0.05, Mann-Whitney test.

Mice in estrus and metestrus were more susceptible to UPEC colonization in the genital tract than mice in proestrus and diestrus (Fig. 3A to C). Mice in estrus (n = 14; 100%) and metestrus (n = 11; 91%) had detectible UPEC colonization in the genital tract (Fig. 3A to C). Although the urine bacterial load was highest in mice during estrus relative to other stages, this difference was not statistically significant (Fig. 3D). Instillation of UPEC strain CFT073 in the vaginal tract did not lead to effective colonization of the urinary bladder (Fig. 3E), regardless of the stage of the estrous cycle. The kidney UPEC load was higher in mice in estrus and metestrus than in those in proestrus and diestrus (Fig. 3F). The tissue bacterial loads of mice in estrus and metestrus did not differ significantly from each other (Fig. 3A to H). During diestrus, compared with proestrus, mice had elevated UPEC loads in the vaginal wash fluid and vagina but not in the uterus (Fig. 3A to C).

UPEC disseminates from the vagina to systemic sites. Mice in estrus also exhibited more systemic dissemination from the urogenital tract to the spleen and liver than those in proestrus (Fig. 3G and H). While there were increases in spleen and liver UPEC loads during metestrus and diestrus, these differences were not statistically significant. UPEC colonization in the kidneys in the absence of bladder colonization is contrary to findings from intravesical inoculation of CBA/J mice with UPEC strain CFT073, where colonization is detected in both the bladders and kidneys. We tested whether UPEC entered the peritoneal cavity from the genital tract before dissemination to kidneys and systemic sites. UPEC was not detected by plate counts in peritoneal lavage fluid from these mice (data not shown). In summary, estrus and metestrus emerged as the stages of the

estrous cycle that were most permissive to UPEC colonization in the murine urogenital tract. Since mice in estrus showed a more consistent colonization phenotype than mice in other stages of the estrous cycle, the rest of the experiments described in this report were performed during estrus.

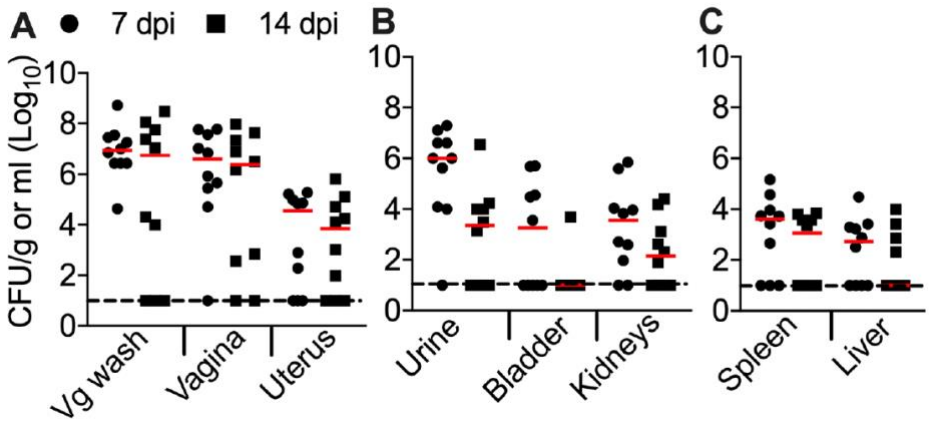

Fig. 4. UPEC colonizes urogenital tract of mice in estrus for at least 2 weeks. Female CBA/J mice (N=10 mice/group) in estrus were inoculated with UPEC strain CFT073 in the vaginal tract. Urine and vaginal lavage were collected at day 7 or 14 post-inoculation, prior to euthanasia. Bacterial loads in the samples were determined by viable counts and normalized to volume/weight of samples. Each symbol corresponds to results from a mouse, and red bars indicate median. Dotted line corresponds to the limit of detection (10 CFU). dpi, day post inoculation, and Vg wash, vaginal lavage.

Temporal changes in UPEC colonization in mice during estrus. To test whether the colonization observed at 1-day post inoculation was transient, temporal changes in bacterial colonization were determined 1 and 2 weeks after inoculation. Mice were inoculated during estrus, and the tissue bacterial burden was determined at later time points. Overall, trends in bacterial loads across the samples were conserved at the 1- and 2-week time points (Fig. 4A to C). There

were no statistically significant differences in the bacterial burdens between the 1- and 2-week endpoints (Fig. 4A to C). We observed an uptick in the median bladder bacterial load at 1-week post inoculation (Fig. 4B), but this difference was not statistically significant. Bacterial loads at these later endpoints were also comparable to that at the 1-day endpoint (Fig. 3 and 4). Collectively, our data reveal persistent colonization by UPEC in the urogenital tract for up to 2 weeks after a single vaginal instillation during estrus.

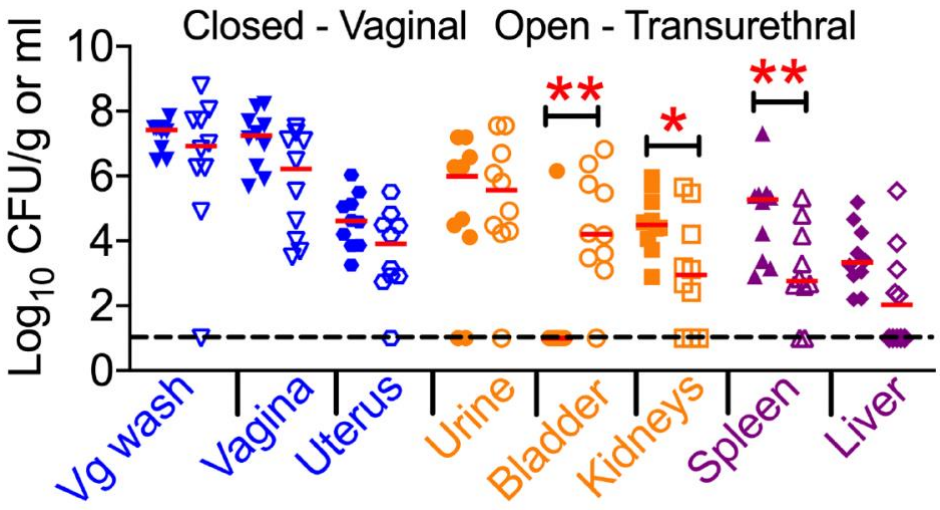

Fig. 5. UPEC colonization after vaginal and transurethral inoculation. Female CBA/J mice (N=10 mice/group) in estrus were inoculated with UPEC strain CFT073 in the vaginal tract (closed symbols) or transurethrally in the bladder (open symbols). Urine and vaginal lavage were collected one day post-inoculation, and mice were euthanized. Bacterial load in the samples were determined by viable counts, and normalized to volume/weight of samples. Each symbol corresponds to results from a mouse, and red bars indicate median. Dotted line corresponds to the limit of detection (10 CFU). Color scheme: blue, genital tract; orange, urinary tract; and purple, systemic sites. *P<0.05, **P<0.01, Mann-Whitney test. Vg wash, vaginal lavage.

Comparison of UPEC colonization in mice inoculated by vaginal and transurethral routes. To further explore the impact of estrus on the colonization of the urogenital tract, we directly compared the outcomes of vaginal and transurethral routes of inoculation. Mice (n = 10 mice/group) in estrus were inoculated either in the vagina or in the urinary bladder with a transurethral catheter, as described in Materials and Methods. As expected, intravesical instillation of UPEC led to a higher bacterial burden in the bladder than vaginal inoculation (Fig. 5). However, mice inoculated in the vaginal tract carried higher bacterial burdens in the kidneys than those inoculated transurethrally (Fig. 5). Both routes of inoculation resulted in comparable colonization of the genital tract and urine with UPEC strain CFT073 (Fig. 5). Increased systemic dissemination from the urogenital tract was observed after vaginal instillation, and this difference was statistically significant in the spleen (Fig. 5). Both vaginal and intravesical instillation of UPEC resulted in genital tract colonization (Fig. 5). In the urinary tract, vaginal inoculation led to renal colonization, whereas intravesical inoculation led to colonization of both the bladder and kidneys.

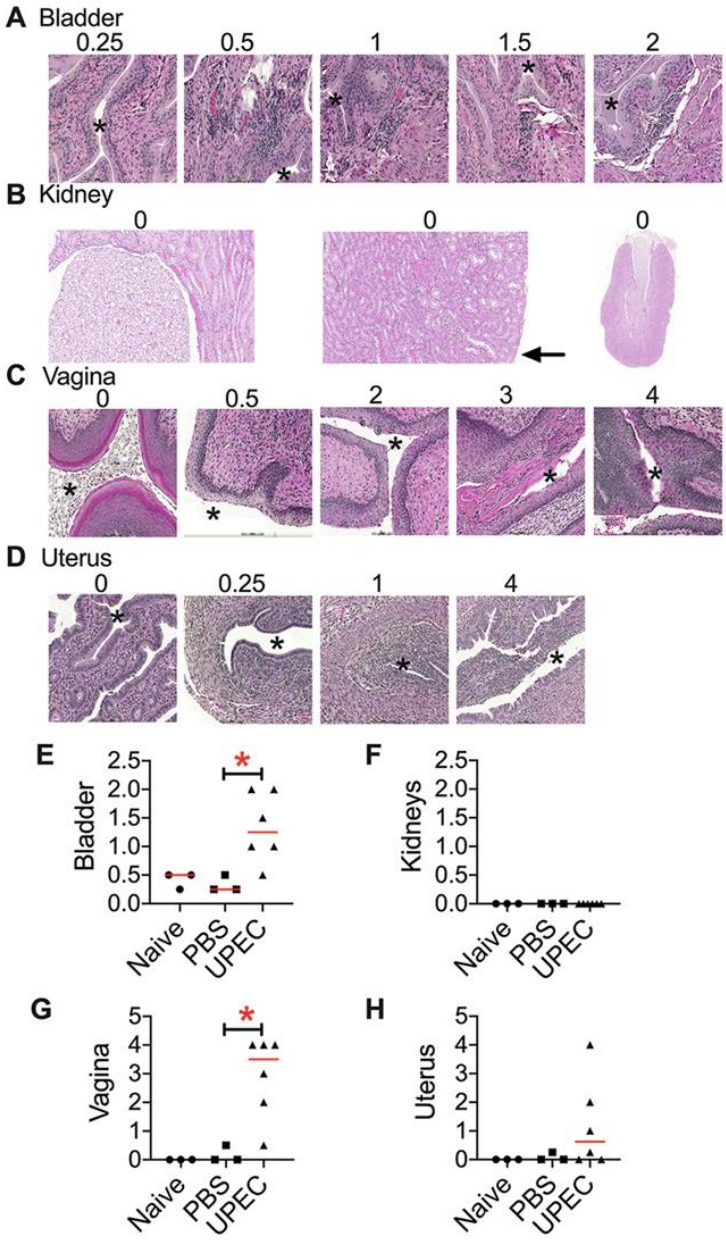

Fig. 6. Histopathological changes induced by UPEC in the urogenital tract. Female CBA/J mice (N=3-6/group) in estrus were inoculated with UPEC strain CFT073 in the vaginal tract. Organs were collected after euthanasia at one day post-inoculation. H&E-stained sections were evaluated by light microscopy. Representative images 636 for each tissue damage score are depicted here (200X magnification, A-D). A low-magnification image of an entire kidney is included (20X, B). Lumen in the bladder, vagina and uterus are indicated with an asterisk. Black

arrow points to renal serosa (B). (E-H) Each symbol corresponds to results from a mouse, and red bars indicate median. *P<0.05, Mann-Whitney test.

Tissue changes in the urogenital tract after UPEC inoculation in the vagina. We next assessed if instillation of UPEC in the vagina resulted in an active infection, defined as a combination of pathogen colonization, inflammation, and damage in the tissues. Levels of key cytokines (interleukin-1b [IL-1b], IL-6, and tumor necrosis factor alpha [TNF-a]) and myeloperoxidase (MPO) (an indicator for neutrophil abundance) in the urine, bladder, kidneys, vagina, and uterus were determined by an enzyme-linked immunosorbent assay (ELISA) (Fig. S1). Levels of these analytes in UPEC-instilled mice were compared to those in phosphate-buffered saline (PBS)-instilled or unmanipulated naive mice at the 1-day endpoint, as reported by us and others (27–29). There were no significant differences in the levels of these analytes as determined by a semiquantitative ELISA between groups at this early time point (Fig. S2). Next, we determined if UPEC colonization in the urogenital tract led to tissue damage by evaluating tissue sections. A board-certified veterinary anatomic pathologist who was blind to the study groups assigned inflammation and tissue damage scores as described in Materials and Methods. UPEC-instilled mice had a higher degree of inflammatory changes in the urinary bladder than the controls (Fig. 6A and E). Bladders from UPEC-infected mice revealed mild-to moderate cystitis, with higher tissue damage/inflammation scores than the control groups (Fig. 6A). We did not observe signs of inflammation and tissue damage in the kidneys despite their elevated bacterial loads, suggesting bacterial colonization that did not progress to an active infection (Fig. 6B and F). UPEC caused a significant increase in pathological changes observed in the vagina (Fig. 6C and G), compared to the control groups. Lesions in the vagina of UPEC-infected mice were consistent with those of

acute purulent vaginitis (Fig. 6C). Although UPEC induced pathological changes in the uterus of some infected mice (Fig. 6D), this change was not statistically significant (Fig. 6H).

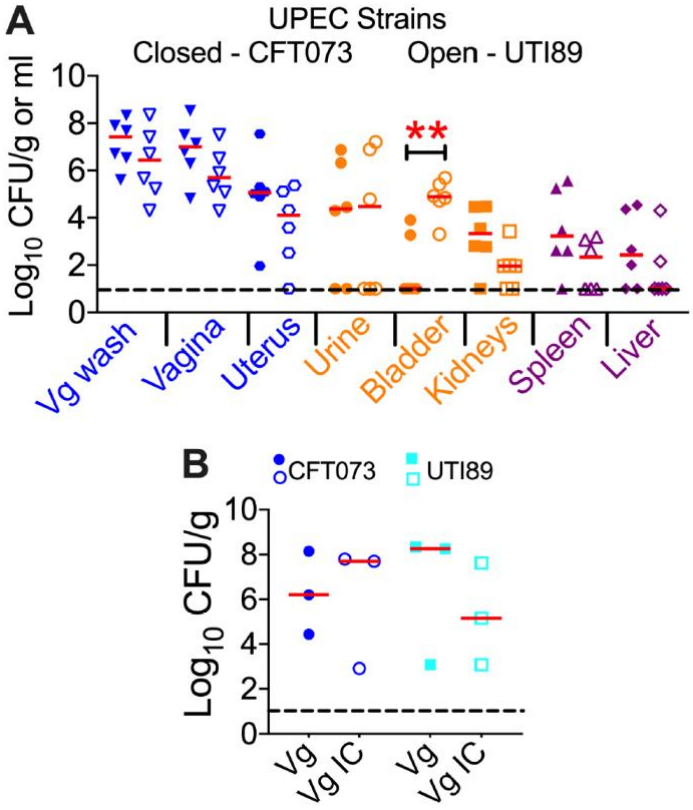

Fig. 7. UPEC strains CFT073 and UTI89 colonize urogenital tract of mice in estrus. (A) Female CBA/J mice (N=6 mice/group) were inoculated with UPEC strain CFT073 (closed symbols) and UTI89 (open symbols) in the vaginal tract. Urine and vaginal lavage were collected one day post-inoculation, prior to euthanasia. Bacterial loads in the samples were determined by viable counts and normalized to volume/weight of samples. Color scheme: blue, genital tract; orange, urinary tract; and purple, systemic sites. (B) Female CBA/J mice (N=3mice/group) were inoculated with UPEC strain CFT073 (blue circles) and UTI89 (cyan squares) in the vaginal tract. Mice were euthanized at one day post-inoculation and vaginas were collected. Total bacterial vaginal bacterial load is depicted in solid symbols. Gentamicin protection assays were performed to detect vaginal intracellular UPEC load (open symbols). Each symbol corresponds to results from

a mouse, and red bars indicate median. Dotted line corresponds to the limit of detection (10 CFU). *P<0.05, **P<0.01, Mann-Whitney test. Vg, vagina, Vg IC, vagina intracellular, and Vg wash, vaginal lavage.

UPEC strain-dependent pattern of urogenital colonization following vaginal instillation. Since UPEC strains are genetically heterogeneous, we evaluated the outcomes of vaginal instillation of two prototypical strains of UPEC (CFT073 and UTI89). Mice (n =6/group in estrus)in both groups showed high levels of colonization in the urogenital tract, urine, and vaginal lavage fluid, except in the urinary bladder (Fig. 7A). Bladder colonization was significantly higher in mice inoculated with UPEC strain UTI89 than in mice inoculated with CFT073 (Fig. 7A). However, in most mice inoculated with UPEC strain CFT073 (4 out of 6), bacterial loads were below the limit of detection in the bladder (Fig. 7A) and were aligned with our above-described findings (Fig. 3 and 5).

UPEC invades vaginal tissue following colonization. UPEC has been demonstrated to invade and establish an intracellular niche in the vagina (24). Here, we tested whether vaginal colonization in mice during estrus leads to invasion and intracellular persistence at 1day post inoculation. Vaginal tissues from mice in estrus instilled with UPEC strains CFT073 and UTI89 were exposed to gentamicin to kill extracellular bacteria on the mucosal surface. Plate counts were performed to determine total and intracellular bacterial loads in the vagina. Our results reveal that vaginal instillation of clinical strains of UPEC results in invasion and intracellular colonization in the murine vagina (Fig. 7B). Importantly, both prototypical strains of UPEC (CFT073 and UTI89) invaded and persisted within vaginal tissue (Fig. 7B).

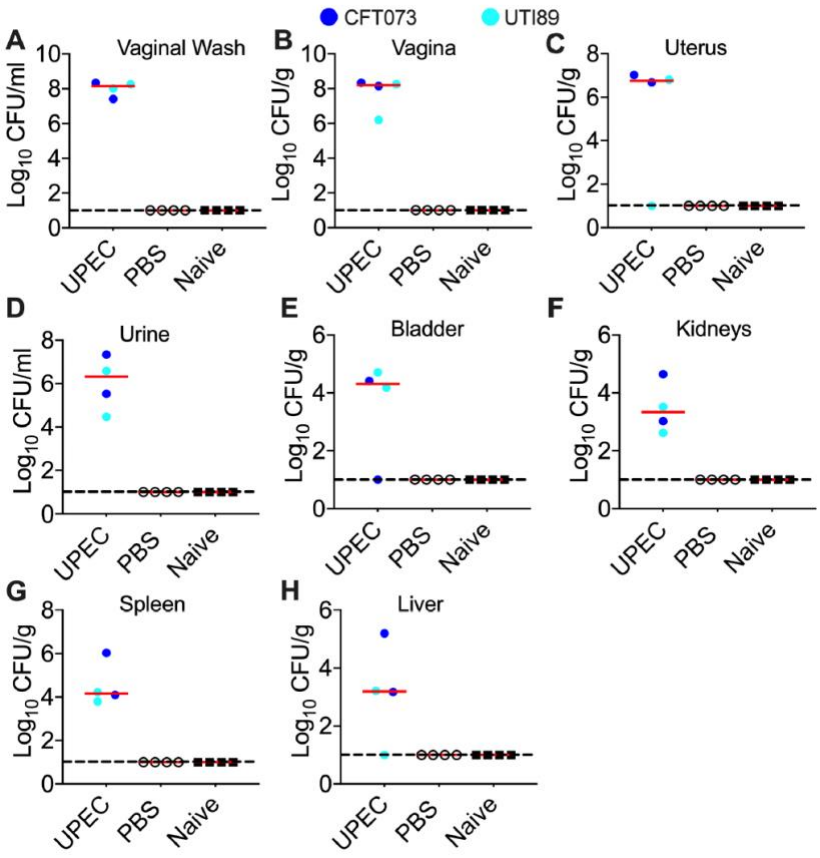

Fig. 8. Contaminated bedding is not a source for urogenital UPEC colonization. Female CBA/J mice in estrus were inoculated with UPEC strain CFT073 (N = 2, blue circles) and UTI89 (N = 2, cyan circles) in the vaginal tract. These mice were co-housed with a PBS-instilled and naïve control mouse such that each cage 659 had two UPEC-instilled and two control mice. Mice were euthanized after collecting urine and vaginal lavage at one day post-inoculation. Organs were collected to determine bacterial load. Each symbol corresponds to results from a mouse, and red bars indicate median. Dotted line corresponds to the limit of detection (10 CFU).

Urogenital UPEC colonization is not due to contamination from bedding. Next, we tested whether vaginal inoculation leads to sustained UPEC colonization or is a result of exposure to contaminated bedding. To address this question, mice in estrus were inoculated with UPEC

strain CFT073 or UTI89 (one UPEC strain/cage) and cohoused with PBS-instilled and naive mice. Animals were euthanized at 1-day post-inoculation, and the bacterial load was determined. Our results revealed that mice that were instilled with clinical strains of UPEC (CFT073 and UTI89) were colonized by UPEC in the genital tract (Fig. 8A to C), urinary tract (Fig. 8D to F), and systemic sites (Fig. 8G and H). However, PBS or naive controls remained UPEC free at all tested sites, indicating that mice are stably colonized by UPEC following vaginal instillation (Fig. 8A to H), ruling out ongoing contamination from bedding as a source of UPEC in the urogenital tract.

DISCUSSION

UTI is among the top bacterial infections encountered by people throughout the world (7, 30). Mouse models of UTI have been used extensively to elucidate host and pathogen factors that are involved in the pathogenesis of UTI and to test the efficacy of therapeutics and prophylactics (15, 18, 19). These models were designed to investigate UPEC colonization, ensuing infection and inflammation in the urinary bladder and renal pelvis, and systemic dissemination from the urinary tract. Existing models either do not involve vaginal inoculation or utilize estrogenized mice (24,31) to probe UPEC vaginal colonization and UTI. The administration of exogenous estrogen has been demonstrated to increase the susceptibility of the female genital tract and kidneys to bacterial infections (32–34). Adult, reproductive-age women represent a preponderance of patients affected by UTI (6, 30). Here, we sought to determine the effect of the stage of the estrous cycle on UPEC colonization in the urogenital tract of adult female mice that were not administered exogenous estrogen. Our approach more closely emulates the physiological changes observed in the adult female genital tract. By comparing various stages of the estrous cycle, our study demonstrates the impact of endogenous changes in the levels of various hormones on UPEC colonization in the murine urogenital tract. The results reported here demonstrate that estrus and metestrus are the most permissive phases of the estrous cycle for colonization of the genital tract, urine, and kidneys and systemic dissemination. Importantly, single inoculation in the vaginal tract leads to colonization for at least 14 days in the urogenital tract and systemic sites. It is well documented that estrus is preceded by elevated levels of estrogen and decreased levels of progesterone, compared to other stages of the estrous cycle in mice (35, 36). Our findings that mice in estrus are more susceptible to UPEC colonization are bolstered by results from estrogenized mice that also demonstrate increased UPEC colonization, compared to controls (24, 31, 34). A protective role for

estrogen against bacterial colonization was detected in ovariectomized mice, which were more highly colonized by UPEC during UTI than the controls (37). Our results are congruent with the established clinical observations that postmenopausal women are at a higher risk for developing UTI (7, 23) and that estrogen has therapeutic value in decreasing UTI incidence and UPEC colonization in this cohort (38–40). Additionally, signaling through estrogen receptors modulates UPEC invasion in a human urothelial cell line in vitro (41). Collectively, these findings reveal an important role for decreased estrogen levels as a determinant of bacterial colonization in the urogenital tract.

Nucleated and cornified squamous epithelial cells are shed from the vaginal mucosa during proestrus and estrus, respectively. The presence of these cell types is routinely utilized to establish proestrus and estrus phases by vaginal cytology, including in this study (42, 43). Exfoliation of superficial epithelial cells in the urinary bladder is a well-characterized host response to UTI (44–46). We hypothesized that exfoliated vaginal epithelial cells would act as decoys to protect against epithelial colonization during proestrus and estrus. However, we observed poor colonization during proestrus and robust colonization during estrus in the vaginal tract, indicating that exfoliated nucleated and cornified epithelial cells exert disparate effects on UPEC colonization.

Stages of the estrous cycle are associated with profound changes in tissue structure, luminal contents, immune cell infiltration, and cytokine levels in the reproductive tract. Histopathological evaluation indicates the induction of inflammation in the vagina and urinary bladder in UPEC-instilled mice (Fig. 6). However, semiquantitative measurements of cytokine and myeloperoxidase levels did not reveal statistically significant differences (see Fig. S2 in the supplemental material). Considering known estrous cycle-dependent changes in cytokine expression in the female genital tract (47), our results suggest that the baseline changes during estrus mask the differences in

cytokine and myeloperoxidase levels among mice in the naive, PBS-instilled, and UPEC-instilled groups. The divergence of the results from cytokine measurements and histopathology evaluation is likely the result of capturing UPEC-induced structural changes in tissues in the latter analyses. The extent to which UPEC colonization affects changes in cytokine expression in the urogenital tract during various stages of the estrous cycle remains to be determined and is a logical extension of this study. Additionally, quantitative ELISAs and flow cytometry should be included in follow-up studies to develop a comprehensive portrait of immune cell and effector changes elicited by UPEC during various stages of the estrous cycle.

UPEC is a collection of genetically heterogeneous bacterial pathogens that are usually derived from E. coli belonging to phylogroups B2 and D (30, 48). A key finding from our study is that genetic differences in UPEC strains determine the potential to colonize the urinary bladder following vaginal inoculation during estrus. UPEC strain CFT073 does not colonize the urinary bladder when inoculated in the vagina, as opposed to strain UTI89, which demonstrates superior bladder colonization. This difference could be attributed to the original sites of infection caused by these prototypical UPEC strains. Strain UTI89 was isolated from a patient with cystitis, whereas strain CFT073 was isolated from a patient with pyelonephritis and bacteremia (49, 50). UPEC strain-specific effects should be considered during the application of the model described in this report.

The vagina has been proposed as an intermediary niche for UPEC during its transmission from the gut to the urinary tract. UPEC strains are known to establish intracellular reservoirs in the bladder epithelium, and these reservoirs have been implicated in the development of recurrent UTI (46, 51, 52). Recently, Brannon et al. demonstrated the presence of intracellular UPEC in vaginal epithelial cells from patients suffering from recurrent UTI, highlighting the importance of

the vaginal epithelium as a niche in the pathogenesis of UTI (24). They also established a mechanism by which UPEC invades and forms intracellular reservoirs in human vaginal epithelial cells (24). Our study demonstrates that UPEC stably colonizes cycling, adult female mice that are not treated with exogenous estrogen. Here, we demonstrate that UPEC strains CFT073 and UTI89 invade vaginal tissue by using gentamicin protection assays in mice during estrus. Further studies are required to evaluate the cellular localization of UPEC in this mouse model. There is the potential for reinoculation of UPEC into the urogenital tract from contaminated bedding that could be mistaken for stable colonization. Cohousing of UPEC-instilled and control mice demonstrates that UPEC colonizes mice in estrus and is not reintroduced from the bedding since the controls remained UPEC free (Fig. 8). The model reported in this study could be applied to interrogate mechanistic questions on the role of the vagina as a reservoir for pathogens affecting the female urogenital tract under physiological conditions.

Despite the comparable anatomic structures of human and mouse vaginas, notable differences have also been described. The vaginal microbiomes are profoundly different between these hosts, with humans harboring a Lactobacillus-predominant microbial community that leads to an acidic pH from puberty through menopause (53). The mouse vaginal microbiome is typically rich in Streptococcus, and the pH of vaginal secretions is near neutral (53, 54). There is also an increasing interest in understanding the role of the vaginal microbiome in UTI outcomes and developing vaginal microbiome-based approaches to deter UPEC colonization (55–57). Therefore, the results from this report and others that utilize a vaginal route of inoculation (31) must be interpreted while being cognizant of the species-specific differences. An avenue to refine the model presented here would be to develop and utilize mice with a humanized vaginal microbiome.

While establishing a simple and physiological murine model of female urogenital tract colonization, our findings also raise important questions for further investigation. Here, we utilized adult female mice to test the effect of the stage of the estrous cycle and associated changes in hormone levels on UPEC colonization. Outcomes of vaginal instillation of UPEC in prepubertal and postmenopausal mice should be evaluated to understand the role of reproductive status in determining susceptibility to urogenital infections. We used CBA/J mice since they develop UTI with a more robust renal involvement. The effect of the genetic background of a mouse strain on the outcomes of urogenital infection is another question that needs to be tested in future studies. In summary, our findings demonstrate that mice in estrus are highly susceptible to UPEC colonization of the genital tract and kidneys along with systemic dissemination following instillation in the vagina.

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AUTHOR CONTRIBUTIONS

S.S. conceived and designed this study; C.K.R., P.S., B.H., and S.S. performed experiments; C.K.R., P.S. L.G.A., and S.S. analyzed data; C.K.R. and S.S. wrote the manuscript, with input from all authors.

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CONFLICT OF INTEREST

We have no conflict of interest to report.

CHAPTER III

CONCLUSIONS AND FUTURE RESEARCH EFFORTS

UPEC is an important pathogen of humans, and animals including dogs and pigs, that can cause a multitude of health issues for those susceptible to UTI. It still remains one of the most common human pathogens that is frequently present inside and outside of hospitals settings. Having a full understanding of the epidemiology and pathogenesis behind the mechanism of UPEC will help create valuable information in the development of infection, resistance, and treatment options for scientists, epidemiologists and partitioners working hard to combat the disease.

How we study the different effects of a UTI in a laboratory often sets the stage for success in an applicable clinical setting. Use of appropriate animal models is critical to harness the translational potential of laboratory research. The ability to utilize instillation methods such as urethral catheterization has been a very prominent part of research efforts to successfully imitate the human pathogenesis of the UTI. However, this method has its limitations, including the use of anesthesia and most importantly the bypassing of a vital organ, the vagina. Bypassing the vagina eliminates a very important step in the ascent of UPEC that occurs during a natural infection in humans. Development of a new vaginal inoculation model with increased construct validity will help to promote a more natural way of studying the effects of UPEC in animal models. Such a model provides critical information that cannot be gleaned from the existing murine models of UTI.

The vaginal inoculation model that was developed and reported here represents a major improvement and refinement of the mouse models of human UTI. Findings presented here also

generate further exciting experimental questions utilizing this natural route of UPEC instillation in the urogenital tract. For example, our vaginal inoculation model was able to discern that instilling UPEC strains UTI89 and CFT073 into the vagina created colonization and dissemination to the spleen and liver. However, at this time we are still unsure how this process takes place. After evaluating the peritoneal fluid, and finding no evidence of bacterial presence in the peritoneal cavity, suggests the existence of another route in which UPEC disseminates from urogenital tract to systemic sites. It is tempting to speculate that bacterial dissemination may have taken place through the lymphatics. This is an important ramification of our findings and future studies will explore this in more detail to develop a mechanistic framework for bacterial dissemination from mucosal surfaces to systemic sites. In addition to systemic dissemination of the spleen and liver, we are also curious to know if combining two or more uropathogens in the vagina would lead to comparable systemic colonization. As we have previously discussed in the Introduction chapter, in humans the vaginal microbiota usually creates a conducive environment for infection and colonization is one that has a diverse vaginal microbiome with multiple species of UPEC present. It would be interesting to see the results of having more than one UPEC species present in the vagina of mice and other animal models to discover their overall host response.

Lastly, it will be important to uncover the role and advantages of *Lactobacillus spp.* present in the vaginal microbiome of healthy women. *Lactobacillus spp.* is associated with a healthy microbiome in women who do not frequently or ever experience a UTI. We now understand that this is not a common commensal organism in the murine vaginal tract, and in further experiments for treatment options this could be implemented to promote a healthier vaginal environment, and potentially reverse the consequences of UPEC, but further studies will need to be conducted to answer this inquiry in mice and other animal models used in UTI research. In conclusion, the

vaginal inoculation model developed in the CBA/J mouse implemented a refined technique to more closely emulate UPEC interaction with urogenital tract that occurs during UTI in humans. This advancement to the mouse model of UTI is expected to significantly help the ongoing research efforts to develop novel approaches to prevent, diagnose and/or treat UTIs.

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