Strengths and Limitations of Model Systems for the Study of Urinary Tract Infections and Related Pathologies

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SUMMARY	351
INTRODUCTION	351
ETIOLOGY OF UTIs	352
THE LIFE CYCLE OF UPEC	352
MODEL SYSTEMS FOR DEFINING THE PATHOGENICITY OF UPEC AND RELATED BACTERIA	355
Humans and Other Primates	355
Cell Culture	356
Murine	357
Swine	358
Avian	358
Zebrafish	358
Nematodes	359
CONCLUDING REMARKS	359
ACKNOWLEDGMENTS	359
REFERENCES	359
AUTHOR BIOS	367

SUMMARY

Urinary tract infections (UTIs) are some of the most common bacterial infections worldwide and are a source of substantial morbidity among otherwise healthy women. UTIs can be caused by a variety of microbes, but the predominant etiologic agent of these infections is uropathogenic *Escherichia coli* (UPEC). An especially troubling feature of UPEC-associated UTIs is their high rate of recurrence. This problem is compounded by the drastic increase in the global incidence of antibiotic-resistant UPEC strains over the past 15 years. The need for more-effective treatments for UTIs is driving research aimed at bettering our understanding of the virulence mechanisms and host-pathogen interactions that occur during the course of these infections. Surrogate models of human infection, including cell culture systems and the use of murine, porcine, avian, teleost (zebrafish), and nematode hosts, are being employed to define host and bacterial factors that modulate the pathogenesis of UTIs. These model systems are revealing how UPEC strains can avoid or overcome host defenses and acquire scarce nutrients while also providing insight into the virulence mechanisms used by UPEC within compromised individuals, such as catheterized patients. Here, we summarize our current understanding of UTI pathogenesis while also giving an overview of the model systems used to study the initiation, persistence, and recurrence of UTIs and life-threatening sequelae like urosepsis. Although we focus on UPEC, the experimental systems described here can also provide valuable insight into the disease processes associated with other bacterial pathogens both within the urinary tract and elsewhere within the host.

INTRODUCTION

Urinary tract infections (UTIs) occur when pathogenic microbes colonize typically sterile niches within the urethra (urethritis), bladder (cystitis), ureters (ureteritis), or kidneys (py-

elonephritis). These infections are among the most common infections experienced by humans, affecting more than 8 million people annually in the United States with associated costs exceeding \$2 billion (1–4). UTIs affect individuals of all age groups and both sexes, although they occur most frequently in otherwise healthy women. It is estimated that one in three women will suffer from a UTI by the age of 24 years, with 50% of women experiencing a UTI at least once during their lifetime (1, 5). Compounding the burden of these infections is their high rate of recurrence. For an individual with a UTI, there is a 25% chance that the infection will recur within 6 months, regardless of antibiotic intervention (2, 5). The probability of recurrence over a 12-month period increases to 46%. Individuals with anatomical abnormalities of the urinary tract and patients with urinary catheters are exceptionally prone to UTIs and may develop complicated chronic infections that can be extremely difficult to eradicate.

Cystitis, which is by far the most common type of UTI, is associated with increased urinary frequency and urgency, pain during urination, cloudy or bloody urine, urine with a strong or foul odor, and discomfort in the pelvic region. A low-grade fever can coincide with cystitis but is more frequently associated with pyelonephritis. A patient suffering from pyelonephritis can have any or all symptoms associated with cystitis in addition to costovertebral flank pain, nausea, and vomiting. Pyelonephritis can also lead

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to renal scarring and diminished kidney function, particularly in young children (6, 7). Episodes in which bacteria disseminate from the kidneys into the bloodstream can be especially serious, resulting in bacteremia and life-threatening sepsis (urosepsis) (4, 8, 9).

Most uncomplicated UTIs are easily treated by using standard prescribed antibiotic regimens, including single-dose treatments with antibiotics like fosfomycin or 3- to 7-day treatments with drugs like nitrofurantoin, trimethoprim-sulfamethoxazole, or pivmecillinam (10, 11). Prophylactic low-dose administration of antibiotics can provide relief and protection for individuals prone to recurrent UTIs (12). However, the effectiveness of nearly all antibiotics used to treat UTIs is declining at an alarming rate as the incidence of antibiotic-resistant bacteria is rapidly increasing across the globe (13–15). To counter the rise of antibiotic-resistant uropathogens, there is a pressing need to better understand the host and bacterial factors that contribute to the establishment, persistence, recurrence, and spread of UTIs.

ETIOLOGY OF UTIS

Strains of uropathogenic *Escherichia coli* (UPEC) are the primary cause of UTIs, being responsible for >80% of uncomplicated community-acquired cystitis and pyelonephritis cases (2, 16). Less common, although still highly problematic, are UTIs caused by *Staphylococcus saprophyticus*, *Klebsiella* spp., *Proteus mirabilis*, *Pseudomonas* spp., and *Enterococcus faecalis* (16, 17). In cases of complicated UTI, occurring in people with anatomical abnormalities or in catheterized patients, the microbial etiology is more diverse. UPEC strains are still the prominent pathogens, but these individuals have a higher prevalence of infections caused by *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and group B streptococci and an increased susceptibility to fungal infections by *Candida* species. Due to its high prevalence, UPEC has been the focus of most research on UTIs and is discussed in detail here.

UPEC and other *E. coli* isolates comprise a highly heterogenic group of Gram-negative bacteria (18, 19). Commensal *E. coli* strains are commonly found in association with the gastrointestinal tract of warm-blooded vertebrates, where the bacteria can establish mutually beneficial relationships with their hosts, providing necessary vitamins (e.g., vitamin K and B-complex vitamins), promoting gastrointestinal homeostasis, and protecting the gut from pathogens (20–23). Pathogenic *E. coli* variants can cause a wide range of diseases both within and outside the gastrointestinal tract. Diarrheagenic strains, like enterohemorrhagic *E. coli* (best known for the O157:H7 serotypes found in contaminated food) and enteropathogenic *E. coli*, can cause gastroenteritis but do not typically cause disease outside the gastrointestinal tract (24). In contrast, UPEC strains appear to rarely cause problems within the gut but can elicit disease when given access to alternative niches such as the urinary tract or bloodstream. UPEC strains originating from the gut are likely the primary cause of UTIs (25), but the factors that promote UPEC fitness within the intestinal tract are just beginning to come to light (26–28).

The ease with which UPEC isolates acquire resistance to commonly used antibiotics contributes to the widespread success of these pathogens. Of deep concern is the recent and rapid intercontinental dissemination of multidrug-resistant UPEC strains, including many so-called ST131 isolates (29–31). In less than a decade, ST131 isolates have become a major cause of UTIs in many

parts of the world, including North America (32, 33). The increasing prevalence of multidrug-resistant UPEC strains within the global community is driving the search for more effective approaches to prevent and treat UTIs, which in turn necessitates the development and use of model systems with which we can discern and mechanistically define bacterial and host factors that impact the infection process.

THE LIFE CYCLE OF UPEC

UPEC typically initiates a UTI by traversing the urethra to reach the bladder (25, 34). The gender bias observed in UTI epidemiology is thought to be in large part attributable to anatomical differences in the male and female genitourinary tracts (2). Specifically, women possess a significantly shorter urethra than men, which greatly reduces the distance that infectious microbes must travel from the urethral opening in order to establish an infection within the bladder. In addition, women can harbor UPEC within the vaginal introitus, which may become contaminated with uropathogens through contact with fecal matter or, in some cases, via sexual interactions (34–36).

Each step leading up to bacterial entry into the urinary tract can be problematic for would-be pathogens. For instance, the natural microbial communities (the microbiota) that persist as commensals within the gastrointestinal tract and vagina can competitively interfere with UPEC survival by limiting space and nutrient availability and by altering host immunity (21, 37, 38). It is probable that phages and toxic products such as colicins produced by commensal organisms also restrict the growth and dissemination of UPEC. Within the vagina, for example, the production of lactic acid and other factors by commensal lactobacilli can damage UPEC strains that are in transit between the intestinal and urinary tracts (39–41). Consequently, a healthy vaginal microbiome can help protect women against ascending UTIs. Interestingly, recent research employing massively parallel sequencing techniques suggests that the urinary tract itself may also be home to a protective microbiota (42).

Once within the urinary tract, UPEC faces a barrage of host defenses and other challenges to its survival (43, 44). Among the most formidable of these is the bulk flow of urine. Shearing forces associated with the release of urine during micturition can dislodge nonadherent and weakly adherent microbes, rinsing bacteria from the body before they have a chance to elicit disease. Incomplete voiding of the bladder greatly increases the risks of bacteriuria and UTI, and these complicated infections are especially problematic for individuals who have anatomical abnormalities or neurogenic bladder due to spinal cord injuries, spina bifida, or other medical conditions (45). Catheterization of these patients is common and can inadvertently facilitate the introduction of uropathogens into the bladder.

In uncomplicated UTIs, uropathogens can resist expulsion from the urinary tract via elaboration of adhesive molecules (adhesins) that mediate bacterial attachment to host cells and extracellular matrix components. Mathematical modeling suggests that bacterial attachment to host tissues within the urinary tract is potentially unnecessary, since small numbers of unbound residual bacteria that remain following micturition should be able to multiply fast enough in urine to continually repopulate the bladder lumen as it repeatedly empties and fills (46). Indeed, some asymptomatic-bacteriuria (ABU) isolates seemingly lack the ability to bind host bladder cells, and yet they can still persist for extended

FIG 1 UPEC-associated fitness and virulence determinants. Shown is a sampling of the many different factors that can promote the survival and virulence of UPEC strains within the host. (Left) These factors include iron acquisition systems like enterobactin and salmochelin. Enterobactin is a broadly conserved, high-affinity siderophore that can be inhibited by the host protein lipocalin. To sidestep this host factor, some UPEC isolates produce salmochelin, a glucosylated version of enterobactin that is not recognized by lipocalin. (Right) UPEC strains can secrete a variety of toxins that can disrupt normal host proteolytic cascades, membrane trafficking, cytoskeletal rearrangements, inflammatory responses, and survival pathways. Indicated here are some of the effects of host cell intoxication by alpha-hemolysin (HlyA), secreted autotransporter toxin (Sat), and cytotoxic necrotizing factor 1 (CNF-1). (Bottom) UPEC can bind host cells and tissues via multiple adhesins. These include Afa/Dr adhesins (which bind integrins, collagens, or CD55), S pili (which bind α -sialyl-2,3- β galactoside-containing receptors), F1C pili (which bind lactosylceramide-containing receptors), type 1 pili (T1) (which bind numerous factors, including $\alpha_3\beta_1$ integrins and UP1a), and P pili [which bind Gal α (1-4)Gal β moieties of glycosphingolipids].

periods at high levels within the urinary tract (47). However, most UPEC isolates appear to require adhesins to effectively colonize the urinary tract.

UPEC isolates encode dozens of adhesins that vary in their receptor specificity and whether or not they are assembled into pili (also known as fimbriae) (34, 48–50) (Fig. 1). One of the most frequently studied adhesins, which is expressed by nearly all UPEC isolates, is the type 1 pilus-associated protein FimH. This adhesin can bind a variety of mannose-containing host membrane glycoproteins as well as components of the extracellular matrix (51). Upon entering the bladder, UPEC encounters a thin glycosaminoglycan-rich glycocalyx that is in loose association with the terminally differentiated superficial umbrella cells that comprise the outer layer of the urothelium (52–54). The apical surface of each umbrella cell is highly enriched for a group of integral membrane glycoproteins known as uroplakins, which are assembled into quasicrystalline arrays of hexagonal complexes (55, 56). Together with underlying layers of less-differentiated epithelial cells, the uroplakin-coated umbrella cells create an exceptionally strong permeability barrier. FimH can mediate bacterial interactions with one of the uroplakins, UP1a, as well as with

several other host receptors that may be situated on and within the urothelium (57–62). Although UP1a is doubtlessly the most abundant receptor available to type 1 pilated UPEC on the urothelial surface, other receptors and other adhesins likely come into play as UPEC encounters changing environments and host cell types during the course of infection.

In addition to promoting bacterial attachment to host cells and tissues, several of the adhesins encoded by UPEC isolates can also stimulate the development of biofilm communities. Biofilms consist of bacterial cells encased within a complex extracellular matrix that can include proteins, nucleic acids, and polysaccharides like cellulose. UPEC-associated adhesins that have been implicated in the formation of biofilms include type 1 pili, F1C pili, S pili, and F9 fimbriae as well as the adhesive autotransporter proteins Ag43, UpaG, UpaB, and UpaH (63–70). Flagella and thin aggregative amyloid fibers known as curli can also promote biofilm formation by UPEC (63, 64, 67, 70). The development of biofilms within urinary catheters and in association with host tissues can render UPEC less sensitive to antibiotics and host defenses like neutrophils (70, 71).

The establishment, persistence, and dissemination of UPEC

FIG 2 Multiple fates for UPEC in association with the urothelium. Interactions between the type 1 pilus-associated adhesin FimH and the sugar side chains of receptors like UP1a or $\alpha_3\beta_1$ integrins can stimulate actin cytoskeletal rearrangements leading to the internalization of UPEC. Bacteria are then either trafficked back out of host cells or taken into membrane-bound compartments that are similar to late endosomes or early lysosomes. Within bladder cells, UPEC can remain in a nonreplicating state, forming quiescent reservoirs that are protected from host defenses and antibiotics. Intravacuolar growth of UPEC is restricted in part by the host actin cytoskeleton, which is dense within the immature epithelial cells of the bladder. Within terminally differentiated umbrella cells, where F-actin is localized primarily at basolateral surfaces, UPEC can break into the host cytosol and subsequently multiply to form IBCs. These bacterial communities are not long-lived and are most apparent during the acute phase of a UTI. The disruption or exfoliation of IBC-containing umbrella cells, as well as the transient formation of filamentous bacteria, can facilitate the efflux and dissemination of UPEC. Bacteria released from within IBCs can infect neighboring host cells, including the immature urothelial cells that appear to serve as the primary home for persistent UPEC reservoirs within the bladder. Released bacteria and exfoliated bladder cells that are flushed from the urinary tract with the flow of urine likely facilitate the spread of UPEC between hosts.

strains within the urinary tract are aided further by the ability of these pathogens to invade host epithelial cells (Fig. 2). Among the dozens of adhesins that are encoded by UPEC, type 1 pili and Dr/Afa fimbriae have been implicated most often as mediators of host cell invasion (51). The type 1 pilus adhesin FimH promotes UPEC entry into bladder cells by stimulating actin cytoskeletal rearrangements that cause the host cell membrane to zipper around and envelop adherent bacteria (72). Host receptors for FimH and other UPEC-associated factors that have been implicated as mediators of host cell invasion include integrins (58, 73, 74), the complement regulatory protein CD46 (75, 76), the complement decay-accelerating factor CD55 (77–79), and the flagellin sensor Toll-like receptor 5 (TLR5) (80, 81). Following entry into host bladder cells, most UPEC cells face one of three fates: (i) they can be quickly returned back to the extracellular environment (82, 83), (ii) they can enter into a nonreplicating quiescent state (83–85), or (iii) they can multiply, forming large intracellular bacterial communities (IBCs) (83, 86, 87). The superficial umbrella cells of the bladder are the initial targets of host cell invasion by UPEC, but the exfoliation of these cells and the influx of neutrophils during the course of a UTI can provide UPEC with access to deeper layers of immature host cells within the urothelium (83, 88, 89).

Within both mature and immature bladder epithelial cells, internalized bacteria that are not immediately expelled are trafficked into membrane-bound compartments that have characteristics of late endosomes and early lysosomes (84, 85, 90). Specifically, these UPEC-containing vacuoles incorporate the host proteins CD63 and LAMP-1 and the lipid lysobisphosphatidic acid (LBPA) but lack the protease cathepsin-D. It is within these compartments, enmeshed within host actin filaments, that UPEC may persist in a nonreplicating state for indeterminate periods that can last for several hours to weeks and perhaps longer (83, 84, 91). Ongoing research indicates that these quiescent intracellular bacteria may serve as reservoirs for the recurrent, or relapsing, UTIs that plague many individuals despite the use of antibiotics (89). Intracellular pathogens are protected from antibiotics due to the permeability

barrier function of the urothelium and the inability of many antibiotics to effectively cross host membranes (92). The quiescent bacteria within bladder epithelial cells are also likely more tolerant of antibiotics since most antibiotics affect only replicating microbes.

Changes in both host cholesterol levels and the actin cytoskeleton can stimulate the transition of internalized UPEC from a quiescent state to a rapidly dividing pool of microbes within bladder cells, forming IBCs that can contain several thousand bacterial cells (84, 93). Bacteria present within IBCs are protected from infiltrating phagocytes and are possibly less sensitive to antibiotic treatments (87). During IBC development, many of the normally rod-shaped bacteria transiently assume a filamentous morphology, with some attaining lengths of well over 150 μm (83, 94). These filamentous bacteria are resistant to phagocytosis by infiltrating neutrophils and can extend for long distances within, and even between, host cells. IBCs likely do not serve as long-lived reservoirs for UPEC within the urinary tract, as host cells containing IBCs will eventually rupture or are shed and cleared with the flow of urine (83, 87, 95). The release of UPEC from IBCs as filaments or as single rods can promote pathogen spread both within the urinary tract and, potentially, between hosts. Cycles in which host cell invasion by UPEC is followed by stages of IBC formation, bacterial quiescence, and resurgence may help drive the development of chronic and recurrent UTIs.

In addition to adhesins, UPEC survival within the urinary tract is also facilitated by the expression of various toxins, chelators, and other factors that allow the bacteria to sequester nutrients and essential metals away from the host, modulate host immune responses, and disrupt tissue barriers (a few examples are shown in Fig. 1) (96–99). However, no single set of virulence factors that is required by all UPEC isolates to cause disease has been identified. Rather, UPEC strains appear to rely upon an assorted array of factors that may act redundantly, dependent upon the niche occupied and the specific environmental stresses present. Although the life cycle of UPEC is not fully understood, it almost certainly involves bacterial exposure to various environmental stresses as

FIG 3 Model systems for investigating the pathogenesis of UPEC and related pathogens. Some of the key pros and cons for each system are indicated.

the pathogens traverse niches both within and between human and animal hosts. For example, UPEC hosts may include pets and farmed poultry and pigs, with time spent between hosts in soil or wastewater or in the meat department of the local grocery store (100–110). The capacity of UPEC and related pathogens to inhabit such a diverse range of niches has helped prompt researchers to develop a variety of model systems to understand the virulence potential, adaptability, and evolution of these ubiquitous pathogens.

MODEL SYSTEMS FOR DEFINING THE PATHOGENICITY OF UPEC AND RELATED BACTERIA

Model systems employed to study the pathogenic behaviors of UPEC isolates have incorporated the use of humans and other primates, rodents, pigs, chickens, zebrafish, and nematodes in addition to primary and cancer cell lines derived from the bladder, kidneys, or other host tissues (Fig. 3). These model systems act as approximations of natural human infections and allow various aspects of the infectious process to be dissected in controlled settings. Thanks to advances in sequencing technology and molecular biology techniques, the genomes of multiple UPEC strains are known and can be manipulated fairly easily by using molecular genetic approaches to generate isogenic mutants and recombinant

strains. Pairing molecular and genetic techniques with model systems allows researchers to delineate key bacterial and host factors involved in the pathobiology of UTIs, with a major goal being the identification of novel therapeutic targets. Although our focus here is on UPEC, it should be noted that many of the model systems discussed in the following sections have also been valuable for analyzing bacterium-host interactions involving a diverse array of other uropathogens (for a few of many examples, see references 111–117).

Humans and Other Primates

To date, there have been few experimental UTI studies carried out in humans or other primates. This is in large part due to ethical concerns and the high costs associated with studies in which primates or human volunteers are deliberately infected. Primates are generally employed as part of preclinical trials aimed at testing the efficacy and safety of vaccine and drug candidates. Human studies often involve epidemiological analyses, with the primary aims frequently being to correlate UPEC strain characteristics with patient history and disease information. These studies can be either retrospective or prospective, often centered on the phenotypic and genotypic analysis of bacteria recovered from urine samples provided by human volunteers as well as genetic analysis of the hosts

themselves. This sort of work has highlighted the increasing prevalence of multidrug-resistant isolates such as ST131 strains and revealed emergent patterns in the types of antibiotic resistance genes and virulence factors carried by UPEC isolates (32, 118–126). For example, analysis of bacterial isolates recovered from the urine of children with febrile UTI indicated that UPEC strains that express type 1 pili cause more severe clinical symptoms than pathogens lacking these adhesins (127). These findings were subsequently verified in mouse and cell culture-based infection models (128, 129).

More recently, techniques like transcriptome sequencing (RNA-seq) have allowed researchers to obtain transcriptional profiles of UPEC isolates recovered directly from the urine of human patients with UTIs. This work indicated important roles for ion transport systems and anaerobic metabolic processes in UPEC within human urine but was limited in its ability to analyze tissue-associated pathogens (130). Still, the results suggested a number of bacterial factors that may be of value as therapeutic targets. Specifically, it was noted that a copper efflux system was highly up-regulated in UPEC isolates from human urine, spurring follow-up experiments in which it was shown that copper supplementation can significantly decrease UPEC colonization in a mouse UTI model (130).

Analysis of human-derived samples is especially useful as a means to validate and expand observations made in other experimental systems. For instance, careful microscopic examination of urothelial cells that are shed by human patients during the course of a UTI indicates that the ability of UPEC to invade and multiply within host cells is not a mouse- or cell culture-specific phenomenon, as some have argued (131–135). Instead, the capacity of UPEC strains to act as facultative intracellular pathogens likely contributes to their success as human pathogens. The imaging of UPEC in association with intact human bladder tissue is more problematic, as this requires the acquisition of biopsy specimens or whole bladders, which are not easily obtained.

Taking a more host-centric approach, population-based studies have revealed behavioral, situational, and genetic factors that influence host susceptibility to UTI. Some of the key groups identified as being at greater risk for developing UTI include sexually active females, pregnant women, elderly individuals, and catheterized patients (2, 136). In sexually active women, the use of spermicide or antibiotics escalates the risk of UTI, with the latter also increasing the incidence of infections due to drug-resistant pathogens (137–140). Both spermicide use and antibiotics can disrupt the vaginal microbiota, which normally protects against the ascension of uropathogens into the urinary tract (2, 37, 141). Shifts in the composition of the vaginal microbiota may also account for the increased frequency of UTIs in elderly women (2, 142). This phenomenon is in part attributable to the diminishment of estrogen levels that occurs as women age. Pregnancy substantially increases the risk of pyelonephritis but has little effect on the risk of cystitis. Although pregnancy can alter vaginal secretions and the microbiota (143), the elevated risk of pyelonephritis in pregnant women is more likely attributable to pregnancy-induced increases in blood volume and glomerular filtration rates and the decreased contractility of the ureteric smooth muscle (2). These physiological changes create an environment within the urinary tract that is more conducive to the dissemination and growth of uropathogens. UTI during pregnancy can have serious repercussions, contributing to premature birth, low birth weight, pre-

eclampsia, and the need for cesarean delivery (144). In light of these correlations, researchers are developing mouse models to better understand the mechanisms by which localized UTIs can influence intrauterine fetal growth and other adverse pregnancy outcomes (145, 146).

Specific genetic variables that alter host susceptibility to UTI have been more difficult to nail down, although their existence is suggested by the fact that women with a predisposition for recurrent UTIs often have family members with the same problem (2, 44, 147). Susceptibility factors for pyelonephritis include reduced expression levels of interferon regulatory factor 3 (Irf3) or of the chemokine receptor CXCR1 (147). Polymorphic variants of other surface molecules, including certain blood group antigens and the pattern recognition receptors TLR4 and TLR5, may also constitute risk factors, as they can serve as attachment sites for uropathogens or as regulators of host inflammatory responses (147–151). The effects of these and other identified risk factors can be subtle and may not be evident in all studies (152). It is likely that the spectrum of genetic modifiers of UTI susceptibility will continue to expand as researchers learn more about the pathogenic mechanisms and host defenses associated with these infections.

The added complexity of UTI research using epidemiological approaches and samples from human volunteers is assuaged by the opportunity to assess pathogenic processes in a natural environment of direct medical importance. However, this work is not without its drawbacks, which may include, in addition to high costs, the use of avirulent isolates rather than full-fledged pathogens for deliberate infections and the genetic heterogeneity of human volunteers. Consequently, in order to better define the molecular mechanisms of UTI in a more controlled setting, many scientists employ surrogate infection models, keeping in mind how their findings may relate to the situation in human patients.

Cell Culture

Cell culture-based assays have been used extensively to investigate the mechanisms and consequences of UPEC interactions with host cells. These assays typically utilize primary, immortalized, or cancer cell lines derived from the bladder urothelium or from the kidneys (e.g., proximal tubule epithelial cells). Host bladder cells are usually grown as undifferentiated monolayers but can be induced to stratify and partially differentiate when grown with appropriate media on membrane scaffolds in dishes or under microgravity conditions generated within rotary walled vessel bioreactors (153–155). Recently, culture systems that incorporate different types of host cells, such as neutrophils plus bladder epithelial cells, have been established to better approximate the cellular complexity of the inflamed urothelium (156). By using small interfering RNA (siRNA) as well as plasmid and viral constructs, host genes within cultured cells can be specifically silenced or, alternatively, overexpressed, facilitating the functional analysis of host factors that may contribute to the pathogenesis of UTIs (58, 90, 157–159). Pharmacological reagents can also be used to interfere with host enzymes and signaling cascades, but consideration should always be given to dosage and off-target effects as well as potential interference with bacterial processes. Emerging technologies, such as engineered clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 systems, will allow easier disruption and high-throughput functional analyses of specific genetic loci within host cells (160, 161).

The use of cultured eukaryotic cells has been instrumental in the

identification of host receptors that are bound by UPEC as well as the delineation of downstream signaling events that modulate inflammation and the internalization and trafficking of bacteria (58, 72, 77, 90, 129, 157–159, 162–171). Cell culture-based assays are also commonly used to define how secreted toxins and other UPEC-associated virulence factors affect host cell functions and viability (172–180). The ease of use, low cost, and amenability to high-throughput assays, genetic manipulation, and biochemical analysis make cell culture-based systems attractive alternatives to many animal infection models. Recent advances with flow cell technology even make it possible to examine UPEC–host interactions using bladder cell monolayers in the presence of urine flow, mimicking conditions within the urinary tract during an actual UTI (181).

Despite the proven utility of cell culture-based model systems, they cannot fully recapitulate the complexity of the host environment with its myriad cell types, complicated tissue architecture, variable nutrient levels, and teeming host defenses. For example, key features of terminally differentiated superficial umbrella cells, including their multiple nuclei and quasicrystalline arrays of apical uropod complexes, are exceptionally difficult to mimic in cell culture, although notable progress in this area has been made with primary urothelial cells in recent years (154, 182). Therefore, detailing the molecular consequences of UPEC interactions with bladder umbrella cells is currently limited in the context of cell culture-based models. Nonetheless, cell culture models continue to expand our understanding of UTI pathogenesis, oftentimes propelling more insightful follow-up studies in humans and animal model systems.

Murine

The murine UTI model system, which is the predominant animal model for the study of UTIs, usually entails the transurethral catheterization of rodents and subsequent instillation of bacteria directly into the bladder lumen. For consistent colonization between animals, researchers will typically inoculate animals with $\geq 10^7$ CFU, which is likely far higher than the titers that would be present at the onset of a natural UTI. The mouse model is exceptionally pliable and can be modified to assess polymicrobial infections as well as catheter-associated UTIs and chronic cystitis (146, 183–188). Rodents share much in common with humans, including conserved immunological factors and similar anatomical features within the urinary tract (183). While direct instillation of bacteria into the bladder bypasses some steps required during natural infections, this model system still allows detailed investigation of many key aspects of ascending UTIs. Specifically, rodent models have been used extensively for comparative analysis of the fitness and virulence potentials of wild-type and mutant UPEC strains as well as for the examination of host responses to UTI. Bacterial titers associated with the bladder or kidneys can be monitored by plating of tissue homogenates collected at various time points postinoculation or by the use of luminescent bacteria that can be visualized in live animals using biophotonic imaging (189–191). Host responses can be examined by various means, including transcriptional profiling and histological analysis of tissue sections. The use of inbred mouse strains reduces experimental noise arising from host genetic variability, and recombinant genetic engineering technologies allow the generation of whole-animal and tissue-specific gene knockouts.

While the pathogenesis of UTIs in female mice has been studied

extensively, relatively little work has been done to understand the impact of these infections on males. This disparity echoes the unequal effects that UTIs have on females in the human population, in which nearly half of all women will endure at least one UTI, compared with only about 12% of males (1). The longer male urethra is thought to hinder bacterial ascension into the bladder, and in the laboratory, this feature renders male mice technically much more difficult to catheterize. One way around this hurdle is to bypass the need for catheterization by instilling bacteria directly into the bladder lumen by way of surgical inoculation via an incision in the abdominal wall (192). By using this approach, it was recently determined that IBC formation and other key events that occur during the development of cystitis are quite similar in males and females. However, notable differences between males and females were observed at later time points. Specifically, male mice had a much higher incidence of chronic cystitis, defined as persistent bacteriuria and protracted inflammation, and were more likely to develop severe pyelonephritis and renal abscesses. These effects were linked with the higher testosterone levels present in male mice, although the mechanisms by which this androgen fuels UTI progression remain unclear (192). Interestingly, older studies indicate that testosterone promotes the secretion of lysosomal hydrolases into the urine (193, 194), a phenomenon that could potentially affect host defenses, including the barrier function of the urothelium.

The availability of genetically distinct mouse strains as well as many mutant knockout and transgenic mouse lines has enabled researchers to assess the specific impact of numerous host genes and immune cells on the progression and outcome of UTIs (44, 195, 196). Mouse lines that vary in their susceptibility to UTI have helped identify host susceptibility factors and provide a platform for assessing the functionality of bacterial fitness determinants. Consider, for example, a recent analysis of isogenic UPEC mutants that lack the *cya* or *crp* gene (197). These genes enable UPEC to synthesize and respond to the second messenger cyclic AMP (cAMP). *In vitro*, the UPEC cystitis isolate UTI89 requires both *cya* and *crp* to effectively utilize alternate carbon sources like amino acids, the principal nutrients available to UPEC within the urinary tract (96, 197, 198). In broth culture assays, the *cya* and *crp* mutants also have increased sensitivities to the damaging effects of reactive nitrogen species and superoxide radicals, akin to those generated by host inflammatory cells (197). In wild-type mice, the *cya* and *crp* mutants are each impaired in their ability to colonize the bladder, whereas in C3H/HeJ mice, the mutants are no longer defective. Due to mutations in the pattern recognition receptor TLR4 and possibly other immune regulators, C3H/HeJ mice are hyporesponsive to lipopolysaccharide and therefore have very limited inflammatory responses during experimental UTI (196, 199–201). Together, these observations indicate that TLR4-dependent innate host defenses within wild-type mice, and not nutrient availability *per se*, are the major factors that restrict colonization of the bladder by the *cya* and *crp* UPEC mutants (197).

Microscopy has played a particularly important role in defining pathogenic processes in murine UTI models, revealing how UPEC strains interact with host tissues and bringing inflammatory responses into better focus. In one intriguing example, researchers surgically exposed kidneys in anesthetized rats and imaged living, infected tissue by using multiphoton microscopy following direct infusion of fluorescently labeled UPEC (71, 202). This work revealed an unexpected synergism between type 1 pili and other

adhesive organelles known as P pili in promoting UPEC colonization and biofilm formation within the proximal tubules of the kidneys.

Rodents, and mice in particular, have also been used to model systemic infections, mimicking episodes of bacteremia and urosepsis that can arise in human patients as serious complications of UTI (203–208). In these models, UPEC is inoculated directly into the bloodstream or delivered systemically via a subcutaneous or intraperitoneal injection. These approaches sidestep more natural routes in which UPEC disseminates systemically from sites within the urinary tract but have nonetheless provided valuable insight into the pathogenesis of urosepsis and related pathologies.

Murine models of UTI and associated infections have tremendous practical utility, being the major *in vivo* platform for addressing the efficacy and safety of new antibacterial therapeutics and vaccine candidates prior to testing in primates or humans. However, it should be emphasized that not all data obtained from murine studies are directly relevant to human UTIs. For example, by sensing bacterial products like flagellin, Toll-like receptor 11 (TLR11) stimulates host inflammatory responses, resulting in a modest but significant reduction in bacterial colonization of the kidneys in the mouse UTI model system (209, 210). However, in humans, the gene encoding TLR11 is not expressed due to the presence of a premature stop codon within an early exon and is therefore likely inconsequential as a defense against UTIs in human patients. Nonetheless, the identification of a functional role for TLR11 in mice highlights interesting questions concerning the evolution and operative redundancy of host defense factors. In the long run, these sorts of observations may also aid the development of new approaches to augment host resistance against UTIs and other infections.

Swine

Like humans, many domesticated animals are susceptible to infection by UPEC and related pathogens (106, 211–215). Each year, thousands of pigs are diagnosed with pyelonephritis and culled in order to prevent widespread infection and contamination (216). Using porcine infection models, researchers have investigated the spectrum of host responses to pyelonephritis as well as the onset of renal scarring that can occur as a consequence of UTI (216–218). The latter problem is often associated with vesicoureteric reflux (VUR) and is especially problematic in human infants, but as children age beyond 4 years, the risk of renal scarring due to UTI drops precipitously (219, 220). In an *E. coli* infection model using both adult pigs and piglets with VUR, it was found that older animals were not any better protected from kidney scarring than piglets (218, 219). Extrapolating from these data, it was argued that aging does not intrinsically decrease the risk of kidney scarring but rather that children who are inherently more vulnerable to kidney scarring will already have developed scars before reaching the age of 4 years. In susceptible individuals, polymorphisms that affect signaling via TLRs or other host pathogen recognition receptors or that reduce the expression levels of cytokines like transforming growth factor β (TGF- β) may result in aberrant inflammatory responses that lead to increased bacterial growth and dissemination and decreased renal tubular function, ultimately causing host cell death and scarring (6, 44, 221). Bacterial toxins like alpha-hemolysin and adhesins like type 1, P, and F1C pili may initiate and/or exacerbate these pathological events. Although porcine models have proven utility in defining pathogenic

processes during UTI, they currently lack the genetic tractability offered by some other animal model systems and are relatively expensive, precluding the use of large sample sizes. On the other hand, the ability of the porcine UTI model to recapitulate many important aspects of pyelonephritis in large mammals makes it a potentially highly relevant system for the assessment of novel therapeutics in preclinical trials prior to human studies.

Avian

UPEC strains are closely related to another group of bacteria known as avian-pathogenic *E. coli* (APEC), the causative agent of avian colibacillosis (222–225). This similarity suggests that analysis of APEC strains in their natural avian hosts might provide insight into UPEC-associated virulence mechanisms and vice versa. Work addressing this possibility has employed infection models in which UPEC or APEC strains are inoculated into the air sac of adult chickens, given via subcutaneous injection of young chicks, or injected into the allantoic cavity of fertilized chicken eggs (222, 226–228). In some studies, bacterial pathogenicity within the chicken model correlates well with virulence in the mouse urinary tract, highlighting the zoonotic potential of APEC strains (227–229). However, as a model for UTI, chickens and the embryonic chick model have some notable disadvantages. Chief among them is the fact that chicks lack a urinary bladder and likely present UPEC and related strains with stresses and opportunities distinct from those encountered within mammalian hosts. Despite these limitations, the low cost and ease of the embryonic chick model system provide an enticing alternative approach for screening UPEC-associated virulence determinants within a naturally susceptible vertebrate host. Furthermore, because UPEC may acquire many antibiotic resistance and virulence genes from APEC strains via horizontal gene transfer (227, 230), avian infection models might allow researchers to better discern selective pressures that affect the evolution of uropathogens.

Zebrafish

Recently, the teleost fish *Danio rerio*, commonly known as zebrafish, was developed as an additional host model system for the analysis of infections caused by UPEC and related pathogens (231, 232). To initiate infection, zebrafish embryos at 48 h postfertilization (hpf) are injected with UPEC at one of two sites: a fluid-filled sac surrounding the heart, referred to as the pericardial cavity, or the blood, via the circulation valley. Inoculation of the pericardial cavity limits bacterial dissemination within the embryo and serves as a model for localized infection. The introduction of UPEC into the circulation valley leads to the dispersal of bacteria throughout the bloodstream of the embryo, mimicking a systemic, sepsis-like infection. Each route of inoculation is thought to present UPEC with distinct challenges in terms of the types and levels of antimicrobial factors, phagocyte numbers, and nutrient availability (204, 231). At 48 hpf, zebrafish have only innate immune defenses, including antimicrobial peptides, complement, cytokines, TLRs and other microbial pattern recognition receptors, neutrophils, and macrophages (233–237). Homologous host defenses are central to the ability of both mice and humans to effectively resist UPEC (43, 44).

Relative to many other available model systems, zebrafish embryos are inexpensive and readily amenable to genetic manipulation and to medium- to high-throughput screens. Consequently, the growth and survival of large numbers of wild-type and mutant

UPEC strains can be rapidly screened within the zebrafish host, and pathogen effects on zebrafish morbidity and mortality can be readily assessed. Within a single day, hundreds of fish can be injected, with minimal costs, taking up only a rack or two within an incubator. Zebrafish embryos are also transparent, allowing easy, real-time observation of the infection process, including visualization of fluorescent gene expression reporters as well as fluorescently tagged bacteria, proteins, and host cell lineages (207, 231). Heart rate and blood flow are also easily monitored in the transparent embryos, along with bacterial dissemination and the development of necrotic lesions or other anomalies in host tissues. The effects of specific host genes on the infection process can be examined by using multiple approaches common to many model systems, including microarray analysis and RNA-seq. In addition, zebrafish genes can be silenced by using antisense RNA molecules known as morpholino oligomers (231) or specifically knocked out by using transcription activator-like effector nuclease (TALEN)- or emerging CRISPR-Cas9-mediated genomic modification approaches (238, 239). Transgenic zebrafish that express foreign genes under the control of constitutive or cell-specific promoters can also be generated by using relatively straightforward approaches (240, 241).

In some instances, the zebrafish model is able to discern pathogenic phenotypes that are sometimes less clear-cut in mice or other host model systems. For example, the pore-forming toxin alpha-hemolysin (HlyA), which is produced by about 50% of all UPEC isolates, can promote the lysis of red blood cells, potentially freeing much-needed iron for use by the pathogens (98). Alternatively, at sublytic concentrations, HlyA can inhibit host cell inflammatory and survival pathways and indirectly stimulate the degradation of host cytoskeletal components (98, 174, 175, 242). In wild-type zebrafish, the survival of a reference UPEC isolate known as UTI89 is entirely dependent upon the expression of HlyA (231). However, in fish that lack phagocytes due to morpholino-mediated knockdown of the host transcription factor PU.1, UTI89 does not require HlyA for survival or virulence. This finding, along with microscopic imaging of infected zebrafish, indicates that a primary function of HlyA is to incapacitate phagocytes, with effects on red blood cell lysis and iron acquisition being secondary. Interestingly, HlyA is not required by all UPEC isolates for survival in zebrafish, suggesting that these pathogens can utilize various mechanisms to counter host phagocytes.

Despite the many benefits afforded by the zebrafish infection model, there are some caveats to this host system. Foremost is the lack of a mammal-like urinary tract in zebrafish, which prevents specific analysis of UTI pathogenesis. In addition, zebrafish are typically maintained at 28.5°C, notably lower than the temperature in most mammalian hosts. Nonetheless, zebrafish have proven to be remarkably useful for delineating UPEC-associated fitness and virulence factors that are relevant to the infection process in mammals (203, 204, 207, 231, 243). The discovery and functional analysis of both bacterial and host factors of importance to UPEC pathogenesis will likely advance in strides as imaging and genetic approaches in the zebrafish model continue to be developed and improved.

Nematodes

The nematode *Caenorhabditis elegans*, which probably comes into contact with *E. coli* within soil environments, is an attractively facile tool for exploring the virulence potential of UPEC and re-

lated *E. coli* isolates (232, 244, 245). These transparent worms consume bacteria as their main food source, which makes bacterial infections of these animals a trivial task. Once placed onto medium containing a pathogenic bacterial strain, the nematodes will graze and consequently become infected. By monitoring the death rate of worms infected with wild-type UPEC strains or isogenic mutants, the effects of defined loci on UPEC virulence can be ascertained. In addition, the roles of specific host genes during infection can be easily assessed by using RNA interference (RNAi), a process initiated by feeding the worms nonpathogenic bacteria that express double-stranded RNA molecules with homology to host messages of interest (246, 247). Nematodes encode a number of innate host defenses and signaling pathways with analogues in mammals, but the worms lack a true vertebrate-like immune system and have no urinary tract (248). As with zebrafish and embryonic chick models, limitations associated with the use of *C. elegans* as a surrogate host for UPEC may be countered by the low cost, genetic tractability, and amenability to high-throughput analysis.

CONCLUDING REMARKS

The virulence traits associated with UPEC are likely adaptations of genes that evolved long before modern humans arrived on the planet. For example, genetic factors that enabled ancestral *E. coli* strains to better avoid killing by predatory amoebae in the environment may have later been coopted by UPEC to evade professional phagocytes within human hosts (249). Such conservation of function means that information gleaned by analysis of UPEC-associated fitness and virulence factors within one model system may be relevant in other environments. Consequently, the use of surrogate hosts like *C. elegans* and zebrafish, or even larvae of the wax moth *Galleria mellonella* (250–252), is not so tangential to UTI research and has the potential to drive important discoveries in human patients. Findings made by using animal model systems can be complemented and extended in broth culture or on agar plates using genetic and biochemical assays that are designed to examine how UPEC mutants and wild-type strains handle specific stresses such as reactive oxygen species or serum components like complement (197, 243). Mathematical and computer modeling of infectious processes and gene regulation in UPEC provides additional insight into the pathogenesis of UTIs and can suggest new hypotheses to be tested *in vivo* (46, 253, 254). In total, these various experimental approaches have helped define the molecular pathology of UTIs and the virulence potentials of UPEC isolates, highlighting numerous processes and gene products that may one day be of significant value as therapeutic targets.

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