The Role of Bacteria in Urology

Dirk Lange
Kymora B. Scotland
Editors

Second Edition

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Editors
Dirk Lange
University of British Columbia
The Stone Centre at Vancouver General Hospital, Jack Bell Research Centre
Vancouver
BC
Canada

Kymora B. Scotland
University of British Columbia
The Stone Centre at Vancouver General Hospital, Jack Bell Research Centre
Vancouver
BC
Canada

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Preface

Before the publication of the first edition of *The Role of Bacteria in Urology*, there was no single book addressing our understanding of this important topic. The expansion of information and new data on the human microbiome have revolutionized the way we think about infection as well as the effects of bacteria on healthy individuals. The discovery of the urinary microbiome has made it clear that those of us with an interest in urologic issues must also become familiar with this burgeoning field.

As work in this area continues apace, we recognized the need for a new edition of this book. We have completely revised all original chapters to reflect new information and emphasized the current issues of significant interest for each topic. In addition, we have greatly expanded the book with eight new chapters addressing topics ranging from the microbiome in female urology, prostate cancer, kidney stone pathogenesis, to infection and encrustation of urologic devices. We have also addressed new findings on the virome and mycobiome with respect to urologic infection.

These chapters have been contributed by experts and enthusiasts in each field of interest covered, providing the most up-to-date understanding of the interactions of humans and their microbial inhabitants. This work was accomplished as a true group effort with the efforts of everyone from the undergraduate and medical students who have volunteered their time to participate in the research efforts of the Stone Centre at Vancouver General Hospital to the urology residents, fellows, graduate students, and postdoctoral candidates who have assisted their mentors in the production of each chapter. We are proud of the achievement of this book and hope that it will prove an easy-to-read, but deeply informative, primer on what we now know about the role of bacteria in urology.

Vancouver, BC, Canada — Dirk Lange
Vancouver, BC, Canada — Kymora B. Scotland

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Bacteria in the Genitourinary Tract: The Microbiota and Efforts to Address Infection

1

Tina Gao and Kymora B. Scotland

Introduction

Traditionally, any bacteria found in the genitourinary system were generally assumed to be pathogenic in nature. While infectious bacterial species continue to be a concern, we are now also aware of the existence of a substantial collection of bacteria living in a homeostatic arrangement in the urinary tract of otherwise healthy individuals.

In this book, we will address the potential role played by bacteria throughout the genitourinary system. We will discuss the involvement of bacteria in phenomena as varied as recurrent urinary tract infections, stone pathogenesis and prostatitis as well as prostate and bladder cancer. This chapter will characterize the genitourinary microbiome and will include a discussion of two popular strategies for prevention of genitourinary infection: the use of probiotics and cranberry juice.

The Microbiome and Its Significance in Urology

Microbiome, a term initially coined in 2001 by Dr. Joshua Lederberg signifies "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" [1]. The concept of the human microbiome has

T. Gao
The Stone Centre at Vancouver General Hospital, Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada
e-mail: tinagao@alumni.ubc.ca

K. B. Scotland (✉)
University of British Columbia, The Stone Centre at Vancouver General Hospital, Jack Bell Research Centre, Vancouver, BC, Canada
e-mail: kymora.scotland@ubc.ca

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evolved to represent the collective genome of microbial cells harboured by a person [2]. It has been known for decades that microbial cells outnumber native human cells by many-fold in our own bodies [2, 3]. The realization that the composition of our overall genetic makeup was predominantly microbial drove the initiation of large scale microbiome projects to understand the synergistic impacts of our resident microbes [2, 4]. These projects, accompanied with the development of powerful and cost-efficient genome sequencing techniques in recent years have allowed researchers to gain insight on how the human microbiome plays a role in health and disease across multi-organ systems [5].

In 2008, the NIH launched phase one of the Human Microbiome Project with a goal to sequence and characterize the genome of five main microbial communities in the human body – the gut, skin, oral cavity, nasal cavity and vagina [4, 6]. These five major sites were further divided into 18 specific anatomical locations used to capture a comprehensive microbial picture [7]. Samples from these locations were taken from the healthy adult cohort, which comprised of 300 volunteers who met the rigid criteria to be considered a healthy participant [6]. These samples underwent genome sequencing and analysis to create profiles of the specific microbial community [6]. The results of this initial study demonstrated microbial diversity between different anatomical regions with unique characteristics supported by each body habitat, as well as variability between individuals [7]. The ability to use rapid sequencing technology, rather than culture dependent technique enabled subsequent studies to identify additional microbiomes in body locations previously believed to be sterile. These include the urinary tract, conjunctiva, lungs, and the in utero environment, all found to have a unique microbiome with a role in immunity, barrier protection and disease modulation [8–15].

Perhaps the most prolific and widely studied microbiome to date is the gut microbiome. It is the largest microbiome, currently consisting of 9.9 million identified genes and over 1000 species phylotypes [16, 17]. Extensive studies of this complex microbial community have demonstrated the link between gut microbiome and common diseases ranging from IBD to obesity [18–23]. It has been labelled as a "forgotten organ" due to its unequivocal metabolic capabilities, and its far reaching effects on host immune development and autoimmune responses [24–26]. For such a large and crucial system, we ask – what effects does the gut microbiome have on urologic diseases?

Some of the most common urologic diseases are urinary tract infections (UTIs) which are thought to occur when bacteria gain entry into the urethra and ascend towards the bladder and beyond [27]. The urinary tract is at risk of bacterial dissemination from the rectum due to proximity. Multiple studies have supported the fecal-perineal-urethral hypothesis, suggesting that a UTI-causing bacterial reservoir is harboured in the colon [28, 29]. A case control study by Paalanne et al. with a pediatric population demonstrated more *Enterobacter* in the fecal microbiome of children with febrile UTI compared to healthy children, who had more abundant *Peptostreptococcaceae* [30]. This study indicates a possible correlation between the gut microbiome and the incidence of UTI in children. Subsequent chapters will further discuss the effect of the gut microbiome in urologic disease.

Probiotics and Urological Diseases

Probiotics are live microorganisms that confer a health benefit to the host if taken in appropriate amounts [31]. They have gained widespread popularity in the last decade, and are heavily marketed to treat and prevent diseases. In recent years, there have been attempts to introduce oxalate degrading bacteria as a therapeutic method to reduce the incidence of kidney stones [32–37].

Probiotics have also been introduced and studied in the context of urinary tract infections (UTI). Two Cochrane reviews in recent years have studied the efficacy of probiotic treatment in the prevention of UTIs in two populations. The first review analyzed nine RCTs comprised of susceptible patients or healthy people who received various mixtures and doses of oral probiotics [38]. They found that probiotic treatment had no benefit in the reduction of recurrent UTI risk compared to placebo or no treatment use [38]. The other review explored three RCTs that examined the use of intravesical instillation of *E. coli* to prevent symptomatic UTIs in people with neurogenic bladder [39]. The results of that review were inconclusive, as the evidence for symptomatic UTI risk reduction was poor in all three RCTs [39]. A recent 2018 review of the use of Lactobacillus in the prevention of UTIs in women revealed that intravaginal probiotic suppositories demonstrate the highest efficacy and were effective against uropathogens [40]. This method of administration may perhaps be the most protective and hopeful as a UTI prophylaxis.

Probiotics have been shown to potentially alter other urological diseases such as bladder cancer. In 2008, A RCT conducted in Japan compared standard chemotherapy with chemotherapy and 1 year of oral *Lactobacillus casei* in bladder cancer patients after receiving transurethral resection [41]. The results concluded that the group who received the *L. casei* probiotic had a 15% higher 3-year recurrence-free survival rate [41]. Could this be a novel treatment in bladder cancer? The reliability of these findings has been questioned due to the un-blinded design of the study, and the high dropout rate of the study group [42]. This warrants further randomized, double-blinded trials to assess the efficacy of probiotics as a cancer treatment adjuvant.

While it is now clear that probiotics have shown some benefit in several studies, it is important to note that in many cases the mechanisms via which these bacteria mediate healthy host conditions are not well understood. Moreover, there continues to be a lack of clarity with respect to which specific species, in what doses and via which method of administration may be best suited to a particular patient. Nonetheless, there are enough data now to suggest real promise for this field. Probiotics will be discussed in detail in a later chapter.

Cranberry Juice

The prevalence of UTIs is higher in women and men before the age of 60 and highest in females aged 15–29 [43]. Anatomically, the female urethra is shorter than the male urethra, which is thought to make it easier for bacteria to reach the bladder before micturition [43]. Additionally, the female urethra is in close

proximity to the vagina and anus, both locations with known microbial communities [43]. Although usually self-resolving in healthy individuals, UTI complications can cause significant morbidity in the elderly and hospitalized patients. Antibiotic use can quicken symptom resolution, but uropathogens are becoming increasingly resistant [43].

There is a long-standing widespread belief in mainstream society that cranberry juice can help prevent and fight urinary tract infections. This idea was possibly born out of studies in the late 1980s, which suggested that cranberries contained components that prevented *E. coli* adherence to uroepithelial cells of the bladder in vitro [44, 45]. This was consistently demonstrated in subsequent ex vivo randomized control trials (RCT) in the twenty-first century [46, 47]. Over the last two decades, several groups have tried to study the clinical effects of cranberry juice on symptomatic UTI prevention, as proof of this principle would make a huge impact on UTI treatment.

Barbosa-Cesnik et al. in 2011 conducted a double blind randomized placebo-controlled trial to determine if drinking 8 oz cranberry juice daily for 6 months would reduce the incidence of a recurrent UTI in college women (n = 319) [48]. The results showed that there was no decrease in recurrence rate amongst the group of women who drank cranberry juice daily [48]. In another RCT, Stapleton et al. recruited premenopausal women into two study groups that received different doses of cranberry juice daily, and the control group who received a placebo juice (n = 176) [49]. The main outcome was time to symptomatic UTI (with pyuria) in a 6-month period. The results again failed to demonstrate a statistically significant difference between the study and control groups [49]. Recently, Juthani-Mehta et al. achieved similar results when they conducted an RCT with women in nursing homes using cranberry capsules (n = 185). Not only did they demonstrate no significant difference in the number of UTIs, there was also no significant difference in rate of deaths or hospitalizations [50].

On the contrary, several RCT's in 2016 have demonstrated conflicting results, showing significant reduction in UTI incidence with cranberry juice treatment in young women, and cranberry proanthocyanidin extract treatments in a mixed study population [51–53]. Afshar et al. also demonstrated that cranberry juice high in concentrations of proanthocyanidin was effective in preventing UTIs in a pediatric population [54]. Currently, the literature appears to be divided on whether cranberry juice can be a UTI prophylaxis. A 2012 Cochrane review comprising 24 studies found that cranberry products did not reduce the incidence of UTIs compared to placebo or other treatments, and thus cannot be deemed as a recommendation for UTI prevention [55]. This is further conflicted by a more recent meta-analysis that concluded cranberry use reduced the risk of UTI by 26% in healthy women [56]. Perhaps, the answer is not so black and white, and that the effects of cranberry juice depend on patient factors. More rigorously controlled trials with larger sample sizes are currently in the pipeline and will hopefully shed light on this persistent and controversial debate [57].

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Overview of Urinary Tract Infections

2

Justin Y. H. Chan, Kymora B. Scotland, and Dirk Lange

Cystitis

Cystitis is the presence of bacteria confined to the urinary bladder [2]. It is characterized by dysuria, frequency, urgency and cloudy urine, with or without suprapubic pain [2–5]. It is often associated with pyuria and occasionally, with hematuria [2]. The infection can be classified as either uncomplicated or complicated, where uncomplicated cystitis refers to cases in which the host is healthy with no structural or functional abnormalities and is neither pregnant nor exposed to medical devices such as urinary stents or catheters. Approximately 95% of all urinary tract infections (UTIs) are uncomplicated bladder infections [6]. All cystitis cases that do not fit into these criteria are considered complicated [4].

Acute uncomplicated cystitis (AUC) occurs in both men and women, but is primarily present in young, sexually active women. It occurs with a frequency of 0.5–0.7 episodes per person annually [3, 4, 7]. Of those infected, approximately 25% will develop recurrent infections within 6 months, with a significant proportion experiencing a second recurrence within 1 year [4, 8].

Diagnosis is typically based on positive urine cultures in symptomatic individuals. Common pathogens responsible for AUC include: uropathogenic *Escherichia coli* (UPEC), which comprises 80–90% of the cases and *Staphylococcus saprophyticus*, which is responsible for 5–10% of infections [3, 9]. Other uropathogens include: *Enterococci* spp., *Streptococcus agalactiae*, *Proteus mirabilis* and

J. Y. H. Chan
The Stone Centre at Vancouver General Hospital, Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada
e-mail: justinyhchan@alumni.ubc.ca

K. B. Scotland · D. Lange (✉)
University of British Columbia, The Stone Centre at Vancouver General Hospital, Jack Bell Research Centre, Vancouver, BC, Canada
e-mail: dirk.lange@ubc.ca

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Klebsiella spp. [3, 10, 11]. Since treatment of such infections is highly dependent on the mechanisms of action of infective agents, it is important to understand the pathogenesis behind the main culprits of cystitis. Here, we describe the virulence factors utilized by UPEC, *S. saprophyticus*, *Enterococcus faecalis* and *S. agalactiae* in the course of a bladder infection.

Pathogenesis

Cystitis often results from colonization of the vagina and urethra with fecal flora, followed by subsequent ascent of the microorganisms into the bladder [12]. Once inside the bladder, uropathogens find ways to adhere to and infect uroepithelial cells. They can also become internalized by host cells where they proliferate and hide away from host immune responses prior to sequential infection [6].

Uropathogenic *E. coli*

In the case of UPEC, the bacterium expresses an array of diverse virulence factors [6]. A major facilitator of host-cell invasion includes a filamentous adhesive organelle known as Type 1 Pili. These pili are hair-like fibers which are distributed throughout the surface of the bacteria [6]. The pili structure is formed by two adapter proteins, FimF and FimG, and by a mannose-binding adhesin, FimH. FimH mediates bacterial adherence to many host glycoproteins and non-glycosylated peptide epitopes. It is also necessary for the initiation of the invasion process, which involves its internalization into the host cell. Once internalized, UPEC can use the host cells as a protected niche to proliferate and persist, forming aggregations and intracellular bacterial communities. Moreover, the pathogen becomes better shielded from host defense mechanisms as well as from a number of antibiotic treatments which fail to reach the internalized microorganism [6]. It is thought to be the eventual resurgence of these dormant reservoirs that give rise to the significant percentage of recurrent or relapsing cystitis cases [6].

Type 1 Pili are only one method used by UPEC to invade host bladder cells. Other virulence factors that stimulate bacterial uptake by uroepithelial cells include interactions between UPEC Afa/Dr. fimbrial adhesins and host receptors, as well as the activation and subsequent degradation of host Pho GTPases by CNF1. To enhance its pathogenicity, the bacterium also expresses an abundance of diversified virulence factors. Although many virulence factors remain to be identified, UPEC is known to express a variety of fimbrial and afimbrial adhesins for attachment, siderophores for scavenging essential iron from host cells and secreted toxins that alter host cell signaling pathways, modulate inflammatory response and stimulate cell death [6].

Staphylococcus saprophyticus

The second most common bacterium responsible for cystitis is *S. saprophyticus*, a Gram-positive, obligate human pathogen [13]. There are several known virulence

factors of *S. saprophyticus* including extracellular slime, lipoteichoic acids which aid in the adhesion to uroepithelial cells, an adhesive and autolytic protein (Aas) which allows for attachment to uroepithelial cells, adherence factor and haemagglutinin (UafA), collagen- and fibronectin-binding protein (SdrI), surface-associated lipase (SssP), serine-rich adhesin (UafB) and urease [14]. However, despite it being the predominant cause of Gram-positive UTIs, relatively little is known regarding the mechanism it uses to invade the urinary tract or how host cells respond to infection [13]. Recently, using C3H/HeN murine models, Kline et al. were able to demonstrate that *S. saprophyticus* induces the shedding of epithelial cells in the bladder [13]. Additionally, the authors found virulence factors SssP and SdrI to be important for the persistence, but not initial colonization of the bacterium [13]. Another newly identified virulence factor includes the surface protein SssF, which King et al. found to be highly prevalent in clinical isolates and was associated with resistance to the antibacterial activity of linoleic acids [15].

Enterococcus faecalis

Enterococcus spp. bacteria are another leading cause of UTIs and more specifically, catheter associated urinary tract infections (CAUTI) [11]. Within the *Enterococcus* spp., *E. faecalis is* the predominant causative pathogen of UTIs. Currently, the pathogenesis of *E. faecalis* remains to be fully characterized, however several virulence factors have been identified as key players in cystitis [16]. Upon entry into the bladder, *E. faecalis* relies on its Surface Protein (Esp) to mediate adherence and colonization of the bladder [17]. Subsequently, a variety of proteins including collagen adhesin Ace, ArgR family transcription factor AhrC, housekeeping Sortase A (SrtA), housekeeping Sortase C (SrtC) and endocarditis and biofilm-associated pili (Ebp) contribute to establishing a biofilm [18–22].

In addition to biofilm formation, *E. faecalis* is able to persist in the urinary tract by modulating the host immune response. One virulence factor used by *E. faecalis* to evade the host immune system is Aggregation Substance (AS). When expressed, AS promotes internalization of *E. faecalis* by macrophages and subsequently, confers protection against superoxide killing [23–26]. Another virulence factor expressed by *E. faecalis* is gelatinase. Gelatinase allows *E. faecalis* to subvert complement-mediated opsonization and neutrophil recruitment by inactivating C3, C3a, and C5a [27–30]. *E. faecalis* also possesses the ability to dampen inflammatory responses through actions of TIR domain-containing protein in *E. faecalis* (tcpF). TcpF functions by interfering with TLR-Myd88 signaling and in turn, preventing NF-κB -mediated immune responses [31].

Streptococcus agalactiae (GBS)

S. agalactiae or Group B Streptococcus (GBS) is a ß-hemolytic chain Gram-positive bacterium responsible for approximately 160,000 cases of UTIs in the United States annually. Similar to *E. faecalis* and *S. saprophyticus*, the

pathogenesis of *S. agalactiae* is not well defined [11]. In a previous in vitro and in vivo study, *S. agalactiae* was demonstrated to bind to bladder uroepithelium and in these areas, morphological rearrangement in uroepithelial F-actin architecture was noted. This suggests that *S. agalactiae* relies on a cell surface receptor that is responsible for inducing intracellular actin assembly as its site of attachment and initial colonization of the host bladder. However, this exact receptor remains to be identified [32].

Upon establishing infection, the sialic acid moieties of the *S. agalactiae* capsular polysaccharide have been shown to play a role in immune response modulation. An in vitro study demonstrated that GBS sialic acids were able to suppress polymorphonuclear cells (PMN) and reduce PMN pro-inflammatory cytokine production [33].

Interestingly, the primary toxin of GBS, ß-Hemolysin/Cytolysin (ß-H/C) which is responsible for the pathogenesis of *S. agalactiae* in various GBS-related diseases, is dispensable in establishing UTIs [34]. Although dispensable, it is hypothesized that ß-H/C could be responsible for the clinical presentation of GBS-related cystitis. This hypothesis is supported by the fact that ß-H/C was observed to elicit potent pro-inflammatory responses in cultured bladder epithelial cells and in murine urinary tracts [34, 35].

Clinical Management, Treatment, and Preventive Measures

If cystitis is suspected based on patient history and physical examination, treatment can be initiated without any further investigations [36]. Urinalysis for diagnostic confirmation is not needed for patients with acute uncomplicated cystitis [37]. However, with complicated cystitis, urinalysis and urine culture should be conducted to guide medical management [36].

To treat uncomplicated acute cystitis, first-, second- and third- line treatments are antibiotics. First-line treatments are nitrofurantoin, sulfamethoxazole/trimethoprim or fosfomycin. Second-line treatments are cephalexin or cefpodoxime proxetil. Tertiary options include levofloxacin or ciprofloxacin. Phenazopyridine can be used as an adjunct therapy for symptomatic relief of associated dysuria [36].

There are supposedly preventative measures patients can take to avoid the occurrence of cystitis. Primary methods proposed to prevent cystitis include post-coital urination, wiping from front-to-back following bowel movements, use of non-barrier contraception and avoidance of spermicide [36, 38]. The use of long-term, low-dose prophylactic antimicrobials can also be used as a preventative measure, especially against recurrent cystitis [10].

It has been suggested that cranberry products can be used as preventative measures against UTIs however, this notion is controversial. Various randomized control trials (RCTs) have been conducted in attempts to provide definitive evidence on the effectiveness of cranberry products. Results from these RCTs are conflicting as some provide evidence of efficacy, while others fail to show any benefit of using cranberry products against UTIs [2, 39].

The prevention and proper treatment of cystitis is crucial. If cystitis is left untreated or is not treated properly, more complicated infections may arise. One such complication involves uropathogens ascending to the kidneys and causing pyelonephritis [2].

Pyelonephritis

Pyelonephritis is defined as a severe infectious inflammatory disease of the renal parenchyma, calyces, and pelvis [40]. Clinically, it is manifested by the following signs and symptoms: fever, nausea and vomiting, voiding symptoms (urgency, frequency, dysuria) and costovertebral angle tenderness [40]. Women are more likely than men to develop pyelonephritis due to a shorter urethra. Other factors that predispose individuals to pyelonephritis are diabetes, kidney stones, bladder tumors, vesicoureteral reflux and other obstructions to the urinary tract that disrupt the normal flow of urine.

Gram-negative bacteria predominate in causing the disease. In particular, *E.coli* comprise the majority of pyelonephritis-associated bacteria while *Klebsiella* spp. and *Proteus* spp. constitute the second and third most common bacteria [41, 42]. Gram-positive bacteria such *as S. saprophyticus*, *E. faecalis*, *S. agalactiae* and *Mycobacterium tuberculosis* are also implicated in rare cases of pyelonephritis, but are rarely described in literature [41, 42]. Research in past years has focused on two common ways for bacteria to infect the kidneys and the upper urinary tract: the ascending mechanism and the hematogenous mechanism [42]. The ascending mechanism requires the initial migration of uropathogenic bacteria from the opening of the urethra up into the bladder similar to cystitis. The difference is that in pyelonephritis, the bacteria migrate higher up from the pelvic mucosa into the upper urinary tract primarily by the action of bladder reflux and to some extent flagellar-driven bacterial movement. In addition, obstruction of the normal urine flow may also contribute to the retention of contaminated urine in the bladder that may propagate back towards the kidneys. In contrast, the hematogenous mechanism is the seeding of circulating bacteria in the blood (bacteremia) as a result of infection at a site distant to the kidneys [42]. Despite the difference in infection pathways, both mechanisms are used by the same set of bacteria, namely *E. coli*, *Klebsiella* spp. and *Proteus* spp. It is important to note however, that most work describing bacterial virulence mechanisms focus on the more common ascending mechanism.

Mechanisms of Bacterial Pathogenesis

Although *E.coli*, *Klebsiella* spp. and *Proteus* spp. are completely different species, they share similar virulence mechanisms in pyelonephritis [43–45]. In the case of the ascending mechanism, the bacteria originate from the host's own fecal sources [41, 42]. Upon introduction into the urethra, the bacteria can migrate upwards

towards the bladder through the use of flagella [43]. As the pathogens migrate up the lower urinary tract they interact with and attach to uroepithelial cells using a variety surface proteins which include: adhesins, P fimbriae and Type 1 fimbriae [43–48]. These structures allow for the bacteria to remain attached to the uroepithelium and protects the bacteria against urinary flow.

In addition to overcoming urine flow, uropathogens are also able to evade the immune system. Normally, when bacteria ascend to the kidneys, they adhere to renal tubular epithelial cells. This adhesion, along with LPS- TLR4 interaction and the disruption of blood cells by haemolysins, trigger the ceramide signaling pathway and LPS-induced TLR-4 dependent signaling pathways [46, 47, 49, 50]. Activation of these pathways leads to an upregulation of pro-inflammatory cytokines (mainly IL-6 and IL-8) and chemokines (including CC-chemokines MCP-1 and RANTES) which in turn, lead to the recruitment of host immune cells [49, 50].

To overcome this challenge, uropathogens express capsular polysaccharide on their surface. This polysaccharide forms a thick protective layer which prevents their opsonization and phagocytosis by activated complement and macrophages, respectively [43–45]. While bacterial infection is the cause of pyelonephritis, the resultant tissue damage is not necessarily caused by the bacteria themselves, but rather the immune response they activate. Granulocytes in particular cause numerous degenerative changes to renal tubular epithelial cells. The degenerative changes that cells experience are mitochondrial swelling, dilation of endoplasmic reticula, increased electron lucency of the cytoplasm and formation of cytoplasmic vacuoles [51]. Conversely, depletion of granulocytes in a rat model almost completely abrogates renal parenchymal damage with minimal bacterial invasion for up to 40 hours [51].

Since free iron is limited in the urinary tract, some bacteria express haemolysin to rupture red blood cells and thus force the release of iron into the urinary environment. Iron scavenging proteins known as siderophores (enterobactin, yersiniabactin and aerobactin) are then employed by bacteria to sequester this free iron and use it for their own growth [43–45].

Infection with *P. mirabilis* and some strains of *E.coli* are further complicated by the fact that they express urease, an enzyme that breaks down urea into ammonia in the urine resulting in a significant rise in urine pH [52]. This process can lead to ammonia-induced cytotoxicity of the renal epithelium. In addition, the increase in pH triggers the precipitation of magnesium ammonium phosphate and the eventual formation of struvite stones. In some cases, these stones are able to grow rapidly and form staghorn stones, which can block the majority of the kidney collecting system [52].

Differences Between *E. coli* Strains in Pyelonephritis and Cystitis

Pyelonephritis and cystitis typically involve bacterial infection by *E. coli*, thus it is often difficult to identify bacterial mechanisms unique to a particular condition. In one of a few rare comparison studies, it has been observed that pyelonephritis-associated *E.coli* strains often carry two to three copies of the pap gene cluster while cystitis-associated *E.coli* strains only carry one cluster [53]. It has also been shown

that pyelonephritis and prostatitis-causing *E.coli* isolates exhibit more virulence factors than cystitis isolates [53]. In particular, pyelonephritis *E.coli* isolates have higher prevalence of the following virulence factors when compared with cystitis: pap gene cluster (pap A, C, E, F, G) that encodes p fimbriae, aerobactin receptor (iutA), siderophore receptor (ireA), colicin V (cvaC) a toxin that inhibits bacterial growth of other or similar bacterial strains, G fimbriae (gafD), M fimbriae (bmaE), increased serum survival gene (iss), invasion of brain endothelium A (ibeA) and pathogenicity marker (malX) [53].

Other observations include different adhesion and growth rates between *E. coli* isolates from pyelonephritis and cystitis. Pyelonephritis strains were observed to adhere better to uroepithelial cells, more likely to mediate mannose-resistant hemagglutination, and often possess more P fimbriae due to the increased copy number of pap gene clusters per bacteria [54].

With respect to growth rates, pyelonephritis-associated *E. coli* strains are able to establish infection at lower concentrations of bacteria and tend to persist in the bladder, kidney, and urine. As a result, after a 7-day infection period, pyelonephritis-associated *E.coli* strains are found to present in higher concentrations in the kidney as compared to cystitis-associated *E.coli* strains [54]. In contrast, cystitis strains colonize the bladder in higher numbers at an early stage of infection, induce more pronounced histologic changes in the bladder, and are more rapidly eliminated from the urinary tract than pyelonephritis *E.coli* strains [54].

Unfortunately, other groups of bacteria including *Klebsiella* spp. and *Proteus* spp. are much less studied and thus, no comparisons could be found in current literature.

Clinical Management, Treatment, and Preventive Measures

If a diagnosis of pyelonephritis is suspected, urinalysis and urine culture should be conducted. A Gram-stain can also be used to direct treatment. For patients with a severe presentation of pyelonephritis, blood cultures are indicated. Other laboratory tests that should be done include a complete blood count, erythrocyte sedimentation rate and serum C-reactive protein [40].

With regards to treatments, patients with suspected pyelonephritis should be managed with empiric treatment before results from blood and urine culture are received. Empiric treatment is initiated to prevent the progression of disease. For mild-to-moderate pyelonephritis this involves the use of oral antibiotics and an adjunct long-acting parenteral antibiotic. Some appropriate first-line empiric oral antibiotic treatments include: cefixime, ciprofloxacin or ofloxacin. Second-line empiric treatments are either levofloxacin or sulfamethoxazole/trimethoprim. Ceftriaxone or gentamicin can be used as the adjunct long-acting parenteral antibiotic. For severe pyelonephritis, patients should be hospitalized and receive IV antibiotic therapy. There are a number of first-line IV antibiotics available some of which are ceftriaxone, ciprofloxacin, ofloxacin, ampicillin and gentamycin or ampicillin/sulbactam. Second-line treatments include the use of levofloxacin, pipercillin-tazaobactam,

imipenem/cilastatin or ceftazidime/avibactam. Upon identification of the causative pathogen in mild/moderate or severe pyelonephritis, targeted oral antibiotics can be started for the former and IV antibiotics can be started for the latter [40].

Similar to preventing cystitis, a proposed preventive measure for pyelonephritis is to void post-coitus. Further, increasing fluid intake to a minimum of eight glasses per day and improving voiding habits are recommended as methods of primary prevention [40].

Urethritis

Urethritis is a urinary tract condition characterized by the inflammation of the urethra. Signs and symptoms of urethritis include: urethral discharge, urethral irritation or itching, dysuria and arthralgia [55]. In adults, urethritis is mainly of infectious nature and usually transmitted by sexual contact [56]. In fact, one of the most prevalent types of sexually transmitted infections (STI) in men is non-gonococcal urethritis (NGU) [57]. Pathogenic microbes most commonly responsible for NGU include: *Chlamydia trachomatis* (30–50% of NGU cases) and *Mycoplasma genitalium* (10–30% of NGU cases) [57]. Other pathogens found to be implicated in urethritis include *Ureaplasma urealyticum*, *Haemophilus* spp., *Streptococcus* spp., *Gardnerella vaginalis*, herpes simplex viruses, adenoviruses and *Trichomonas* species [57, 58]. Another form of urethritis is gonococcal urethritis (GU) and the causative pathogen is *Neisseria gonorrhoeae* [58]. In men, if the bacteria from NGU and GU are allowed to spread, urethritis may lead to epididymo-orchitis (inflammation of the epididymis or testis) and result in impaired fertility [59].

C. trachomatis exists in two morphological forms: the intracellular reticulate body form (RB) and the (extracellular) elementary body form (EB) [60]. The EB form of *C. trachomatis* is metabolically inactive, but is infectious [61, 62]. It is this form of *C. trachomatis* that is responsible for the initial colonization of the urethra and in turn the development of NGU. When the EB form of *C. trachomatis* enters the urethra, it infects susceptible host cells by using a heparin sulfate-like glycosaminoglycan molecule on its cell surface to bind an unknown host cell receptor [62]. Although the receptor is unknown, it is established that these host receptors are localized to the apical surface of polarized cells, thus genital epithelium are affected by *C. trachomatis* [61]. Following binding, the EB uses a Type 3 Secretion system to translocate bacterial proteins known as Tarp (translocated actin-recruiting phosphoproteins) into host cells, which results in the recruitment of actin and the promotion of its internalization [61, 63]. When the EBs are endocytosed, they are placed into a membrane bound compartment known as an inclusion. In the inclusion, the EBs are further transported into a perinuclear location in the infected cell. Following this internalization process, *C. trachomatis* EBs differentiate into RBs [62]. RBs are metabolically active and are mainly responsible for *C. trachomatis* proliferation via binary fission [60, 62]. In 24–72 hours, the newly generated *C. trachomatis* progeny differentiate into EBs and induce cell lysis in order to escape and infect more cells [61, 62]. In response to *C. trachomatis* infection, the host initiates a Th1 immune response [64]. This immune response is pro-inflammatory and induces cell mediated immunity, resulting in an inflamed urethra. If an extreme Th1 immune response occurs, tissue damage may result [65].

For *M. genitalium* to cause NGU, colonization of the urogenital tract is imperative. When *M. genitalium* is exposed to host urogenital epithelial cells, *M. genitalium* relies on a complex tip structure known as a terminal organelle to adhere to the host cells [66]. On the cytoplasmic side, the terminal organelle is attached to the cytoskeleton structure of the *M. genitalium*. On the apical surface of the terminal organelle are two cell surface adhesins, P140 and P110, which mediate binding to susceptible host cells. Successful binding of *Mycoplasma* to host cells is followed by internalization. Similar to *C. trachomatis*, *M. genitalium* is found to localize to the perinuclear space and replicates by binary fission [67, 68]. When inside the host cell, *M. genitalium* produces a toxin known as MG-186. This toxin is a calcium-dependent membrane associated nuclease, which degrades host cell nucleic acid and provides the *Mycoplasma* with a source of nucleotide precursors for its growth and pathogenesis [68]. A previous study (using lung fibroblast cells) has shown that at 96 hours post-infection, *M. genitalium* lysed the infected cell and released progeny into the surrounding environment [69]. In response to *M. genitalium* infection, the immune reaction produced by the host is largely dominated by polymorphonuclear leukocytes [68]. Lipoproteins found on the cell surface of *M. genitalium* also contribute to the inflammatory immune response. When immune cells interact with these lipoproteins, pro-inflammatory cytokines are produced and in some cases this interaction may lead to necrosis or apoptosis [70].

In cases of GU, *N. gonorrhoeae* is responsible for the infection of urogenital epithelial cells [71]. When *N. gonorrhoeae* encounter urogenital epithelial cells, the bacteria rely on Type IV Pili to adhere to these cells [72]. More specifically, for adherence, the Type IV Pili interact with a human-specific complement regulatory protein 46 (CD46) [72]. Upon adhesion, outer membrane bacterial proteins known as opacity protein adhesins (Opa) proteins may bind onto heparin sulphate proteoglycan and carcinoembryonic antigen-related family of cell adhesion molecules (CEACAM) and allow for the gonococci to be internalized [71–73]. Another bacterial cell surface protein crucial for the infection of the urogenital epithelial cells is porin. *N. gonorrhoeae* porin has been shown to function as an actin-nucleating protein in epithelial cells and in turn, aid in actin-mediated internalization of gonococci into cells [71, 74]. Following internalization by host urogenital epithelial cells, the gonococcus undergoes transcytosis to reach the basilar side [75]. The intracellular processes of *N. gonorrhoeae* remain to be elucidated [71].

When *N. gonorrhoeae* infection is detected by the immune system, a strong pro-inflammatory response is elicited [76]. To further contribute to inflammation, *N. gonorrhoeae* express lipooligosaccharide (LOS). The interaction of urogenital epithelial cells with bacterial LOS has been shown to result in the production of cytokines and chemokines such as TNF-α, IL-1β, IL-6 and IL-8. As a consequence of cytokine and chemokine secretion, polymorphonuclear leukocytes are recruited and inflammation results [71].

Clinical Management, Treatment, and Preventive Measures

When a patient presents with a history and physical exam consistent with the diagnosis of urethritis, a Gram stain is typically used to differentiate between GU or

NGU infections. Nucleic acid amplification tests (NAAT) can then be further used to identify the offending organism [55].

If an individual is diagnosed with GU, dual antibiotic therapy is used as first-line treatment. Dual antibiotic therapy is warranted as individuals infected with *N. gonorrhoeae* are often co-infected with *C. trachomatis.* Ceftriaxone and azithromycin are used as the primary treatment options while the secondary options include either a combination of cefixime and azithromycin or cefotaxime and azithromycin. For individuals diagnosed with NGU, azithromycin or doxycycline can be used as first-line treatments. Second line antibiotics used include: erythromycin base, erythromycin ethylsuccinate, levofloxacin or ofloxacin [55].

As urethritis is transmitted through sexual contact, various preventative measures can be taken. Primary prevention involves providing youth with education on safe sex practices and encouraging sexually active individuals to practice risk reduction strategies. These strategies include use of condoms, abstinence, and limiting the number of sexual partners [55, 77].

Microbiome of the Urinary Tract

In the last several years, there has been a shift of thought regarding the sterility of the urinary system. Previously, the medical and scientific community believed in the century old dogma that states "urine is sterile". This dogma contributed to creating a barrier against research into the microbiome of the urinary tract. In fact, the Human Microbiome Project (HMP) excluded the testing of the bladder because it was thought to be sterile [78]. Another barrier that hampered the research of the urinary microbiome was the traditional bacterial culture methods. Typically, these methods are used to identify fast-growing aerobic pathogens and thus, slow-growing, anaerobic or fastidious bacteria were not previously identified [1, 79]. However, new culture methods like the Expanded Quantitative Urine Culture Protocol (EQUC) have been developed to overcome this obstacle. EQUC requires the plating of more bacteria, use of varied atmospheric conditions and the prolongation of incubation times [79]. In addition to new culture techniques, improvements in molecular biology techniques such as PCR and 16s Sequencing have allowed for the discovery of the urinary tract microbiome [1]. With the advent of these new techniques, characterization of the urinary tract microbiome has now begun. This topic will be further discussed in subsequent chapters of this textbook.

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Pathogenic Mechanisms of Uropathogens

3

Kaylen O'Rourke and Peter A. Cadieux

Introduction

While numerous species of fungi and Gram-positive and Gram-negative bacteria have been shown to cause UTIs, ~80% to 90% of community-acquired infections are due to strains of UPEC [1, 2]. As a result, the majority of UTI research over the past several decades has focused on this organism and the strategies it utilizes to colonize and persist in the urinary tract, despite strong host defences and antimicrobial therapies. This review will cover the most common and well-studied UPEC VFs, with minor references to other factors and pathogens. Importantly, most uropathogens express related homologues and analogues to many of the factors described herein, highlighting the most critical attributes required for any organism to cause a successful UTI. Specific topics discussed are the fimbriated and non-fimbriated adhesins for attachment and host invasion, biofilms and related bacterial communities, exotoxins for host invasion and immune evasion, siderophores for iron procurement, antimicrobial resistance and tolerance, urease production, and bacterial expression changes controlling metabolism, division and cell structure that enhance bacterial survival and the infection process.

K. O'Rourke
ER Department and OR Department, Alexandra Marine and General Hospital, Goderich, ON, Canada
e-mail: korourk9@uwo.ca

P. A. Cadieux (✉)
School of Health Sciences, Fanshawe College, London, ON, Canada

Department of Microbiology and Immunology, Western University, London, ON, Canada
e-mail: pcadieux@fanshawec.ca

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Bacterial Adhesion

The first step in establishing infection in the urinary tract is host cell adherence and uropathogens engage a variety of mechanisms to accomplish this. Many species including the vast majority of UPEC strains produce long extracellular fimbriae containing distal adhesive protein tips that attach to specific bladder epithelial surface receptors [3–6]. This binding initiates the infection process by enabling bacterial colonization, proliferation, host cell invasion and the formation of biofilms and intracellular bacterial communities (IBCs) [4, 5, 7]. Bacterial attachment also permits stabilization, allowing bacteria to resist elimination by micturition [8].

Type 1 Fimbriae

UPEC attachment to the urinary epithelium occurs chiefly through Type 1 fimbriae, a multi-subunit pilus whose adhesive tip lectin, FimH, binds highly mannosylated uroplakin Ia (UPIa) glycoprotein receptors present on the surface of bladder umbrella cells [9, 10]. Type 1 expression is mediated primarily via the orientation of an invertible DNA promoter element *fimS* (fim switch), resulting in bacteria residing in either a fimbriated (ON) or nonfimbriated (OFF) state [11–14]. Studies have demonstrated that Type 1 pili are expressed at different levels within three major urinary tract niches: unbound within the urine, adherent to the luminal surface of bladder epithelial cells (or urinary devices) and housed within the host cell cytoplasm in IBCs. Planktonic bacteria free within the urine are predominantly Type 1 negative due to a phase OFF phenotype induced by urinary factors including high osmolarity and acidic pH [15–17]. While the reduced oxygen tension observed in the urine can also inhibit Type 1 expression [18], at least a portion of this has been shown to be recovered via fumarate and nitrate reduction [19]. Combined with host mannosylated urinary proteins such as Tamm-Horsfall binding up much of the pre-existing pili already on the surface [20, 21], UPEC Type 1 function is virtually non-existent in the planktonic state [22, 23]. However, any UPEC that manage to approach and bind mannose receptors on the bladder cell surface undergo a switch to *fimS* phase ON, and type 1 pili expression greatly increases [17]. This occurs even in the face of the existing urine conditions and leads to improved attachment and colonization. A similar switching also occurs on the surface of implanted urinary devices such as catheters [24]. These devices naturally become coated with urinary constituents including mannosylated proteins, providing strong points of attachment for FimH [25]. Lastly, studies have shown that Type 1 fimbriae are critical to IBC formation and maturation within uroepithelial cells and its expression is upregulated at these sites, potentially driven by oxidative stress [26–28].

In addition to Type 1 expression levels, FimH binding strength has also shown to be important in infection development. FimH variants have been identified and categorized as possessing either low or high binding affinity for D-mannose, with strains typically expressing some degree of equilibrium between the two. This affinity is tied to the rotational ability of mannose-bound FimH such that the greater the

affinity, the longer that FimH will stay bound on the receptor like a movable tether. While high-affinity binding can enhance the strength of bacterial attachment, it also leads to increased coating by mannosylated urinary constituents and improved clearance by the host. Strains exhibiting lower and intermediate binding therefore typically display more overall success in colonization and IBC formation [29].

Once Type 1 attachment occurs a number of physiological changes happen in the host cell, including phosphorylation of the uroplakin (UP) signaling complex, an increase in intracellular calcium levels, and Rho-GTPase activation leading to local actin cytoskeleton rearrangement and UPEC engulfment via a zippering mechanism [30, 31]. It also triggers exfoliation of these terminally differentiated umbrella cells in an attempt to remove infected cells; while this can assist in clearing a significant amount of the invaders, it also exposes the underlying undifferentiated cells to attack [32]. It is therefore important for UPEC to gain access to this underlying layer before substantial exfoliation occurs or block this shedding process via another mechanism.

P Fimbriae

P fimbriae play a significant role in the pathogenesis of pyelonephritis and ascending UTIs [33–36]. Although P fimbriae are not fundamental to the development of cystitis, a synergistic effect between P Type and Type 1 fimbriae has proven to enhance UPEC invasion of renal epithelial cells and establish more serious kidney infections [37, 38]. P fimbriae bind to renal glycosphingolipids carrying gal (α1–4) gal moieties through their distal adhesion protein tip, PapG [36, 39]. Attachment of this receptor releases ceramine, which acts as an agonist of Toll-like receptor 4 (TLR-4) [40]. TLR-4 binding activates the immune response, leading to the production of proinflammatory cytokines/chemokines and neutrophil recruitment [41]. Although this immune stimulation promotes bacterial clearance, the acute phase response triggered also causes tissue damage, producing more UPEC access points and instigating the pain associated with acute pyelonephritis [42]. Furthermore, P fimbrial expression can block certain immune functions such as IgA transport to the renal lumen [43, 44] and the bactericidal effects of neutrophils [45], enhancing UPEC survival. Lastly, P. fimbriae have been shown to enhance colonization of the renal tubular epithelium in a rat model of UPEC UTI, leading to nephron obstruction [38].

S Fimbriae and F1C Fimbriae

While not as prevalent or well-studied as Type 1 or P fimbriae, both S and F1C fimbriae have been shown to contribute significantly to UPEC infections. The two adhesins are considered to be homologues, related genetically and functionally, both binding various endothelial and epithelial cells in the upper and lower human urinary tracts [46–49]. They do, however, express different binding specificities as S fimbriae bind glycoprotein sialic acid residues, glycolipids and plasminogen

[48, 50] while F1C fimbriae bind β-GalNac-1,4β-Gal residues on glycolipids [51]. These binding differences may explain why only S fimbriae exhibit hemagglutination properties and are linked to more serious UPEC-related meningitis and sepsis infections [52].

Non-fimbriated Adhesins

UPEC strains employ a number of adherence factors apart from pili that aid in colonizing the host urinary tract. The Afa/Dr family of adhesins consists of at least five members that bind various host receptors within the urinary tract. All members of the group bind the Dr[a] blood group antigen present on complement regulatory molecule CD55, also known as decay-accelerating factor (DAF) [53, 54]. CD55 is a negative regulator of the complement system and is expressed in modest amounts on urothelial cells. Dr and Afa-3 adhesins also adhere to α5β1 integrin, also known as the fibronectin receptor [55, 56]. UPEC attachment to either CD55 or α5β1 integrin has been shown to stimulate bacterial uptake, thus adding to the number of UPEC factors promoting internalization. Three Afa/Dr members (F1845, Dr and Afa-3) also bind several members of the carcinoembryonic antigen-related adhesion molecules group (CEACAM) family [57, 58], a group of antibody-related glycoproteins expressed on the apical surface of mucosal epithelial cells. Binding of UPEC to these receptors increases host cell "stickiness" as it stimulates the activation of integrins which augment matrix adhesion. This results in reduced exfoliation of infected cells, enhancing UPEC colonization and persistence. Finally, Dr fimbriae are also known to bind type IV collagen, which has been shown in a mouse model of pyelonephritis to aid in UPEC persistence in the interstitial compartments of kidneys [59, 60].

Ag43 and UpaE are surface-bound autotransporters demonstrated to promote biofilm formation and UPEC persistence during UTI [61, 62]. Both proteins have been shown to adhere to multiple host ECM proteins including fibronectin, laminin and several types of collagen [62, 63]. Type one secretion A protein (TosA) is a nonfimbrial adhesin and member of the RTX family of toxins that binds to kidney epithelial cells in both mice and humans, promotes bacterial survival during disseminated infections and increased lethality from sepsis in two animal models [64]. Lastly, studies show that OmpT plays various roles in UPEC pathogenesis, including adhesion, invasion, IBC formation and acute inflammation [65].

[a] There are a number of highly prevalent antigens known as the Cromer blood group antigens that can be found on the host protein Decay Accelerating Factor (DAF), also known as CD55. One of these particular antigens (antigens can also be referred to as "epitopes") is the Driori antigen (known as Dr). Since it was the first Dr antigen identified, it was given the superscript "a". Of the Dr antigens, there is only Dr[a]. Other members of the group possess multiple similar subtypes and as such are labeled a, b, c, etc. (Tc[a], Tc[b], Tc[c]).

Biofilms and Related Communities

Biofilms

Despite the shedding of the umbrella epithelium, many UPEC strains are able to avoid clearance by penetrating deeper into the underlying, undifferentiated bladder cells, allowing the bacteria to form biofilms. Once bacteria attach to a surface they change from a planktonic to a sessile form [66, 67] and begin producing extracellular matrix (ECM). This variable mixture of proteins, polysaccharides, lipids and nucleic acids facilitates host adherence, bacterial aggregation and surface colonization, and encases the organisms in a protected microcolony on the surface of implanted urinary devices, bladder walls, and within bladder epithelial cells [4, 68–71]. As the biofilm colony inside the ECM grows and matures into a three-dimensional structure, bacteria respond to several environmental cues leading to changes in metabolism and the expression of surface molecules and virulence factors. Cells buried deeper within a biofilm typically enter a very slow or even dormant metabolic state. These alterations enhance survival in unfavourable conditions such as pH changes, urinary flow, host immunity and the presence of antibiotics [72, 73]. Furthermore, these environmental changes lead to bacterial detachment from the biofilm, reversion to a planktonic state, subsequent spread and formation of new biofilms, essential to the development of recurrent infections [74]. Many of the aforementioned adhesive factors used for host attachment and auto-aggregation can be linked to biofilm formation. Urinary devices such as catheters and stents are prime locations for biofilms as they become coated almost immediately upon implantation with host urinary constituents [75]. This conditioning film masks the exposed surface and offers a plethora of attachment points with little to no bacterial resistance. Even devices coated and/or impregnated with antimicrobial compounds cannot thwart biofilm development for long. Once biofilms are formed, they are very extremely difficult to remove and typically require the removal of the entire device alongside antimicrobial therapy in order to successfully eradicate the infection [76].

Intracellular Biofilm Communities (IBCs)

IBCs and other intracellular bacteria are considered vital populations contributing to the persistence and recurrence of UTIs [68, 77, 78]. These communities develop within the terminally differentiated umbrella cells of the bladder and can contain over 10,000 organisms per host cell [26]. Cell culture experiments and in vivo mouse models have detailed the IBC cycle, which can begin within 15 min of bacterial inoculation. Upon initial internalization, UPEC are confined within membrane-bound acidic vacuoles resembling late endosomes where they are unable to efficiently replicate [9]. Subsequently, some of the organisms will escape or be expelled from this compartment into the cytosol where they begin to rapidly

proliferate to form large, dense, intracellular aggregates resembling biofilms [27, 79]. Indeed, doubling time has been shown to be less than 30 min during the early stages of development, considering $>10^4$ organisms can be found within an IBC at ~6 h post inoculation [68, 78]. IBCs have been found to be predominantly clonal, meaning that most are derived from a single bacterium [78, 80]. While the initial stages of replication produce predominantly rod-shaped progeny similar to the original parent, shorter more coccoid-shaped organisms begin to dominate the colony over time. This may be to maximize the number of UPEC produced while maintaining host cell integrity [77, 81]. Eventually the host cell membrane becomes compromised and IBC organisms escape into the bladder lumen. Many of these organisms are highly motile and have the potential to invade neighbouring host cells. At this point, some of the organisms also transform into a long, filamentous form due to a lack of septation. This is believed to be a result of the host innate immune response since it does not occur in TLR-4 deficient animals [77]. These filaments are resistant to phagocytosis and are able to extend both within and between adjacent host cells, spreading the infection [77]. mRNA studies have identified numerous genes that are upregulated during IBC formation, most notably several involved in oxidative stress resistance, Type 1 pili expression, galactose metabolism and iron acquisition [82, 83]. While a detailed IBC life cycle in humans has not been elucidated, these communities alongside the presence of filamentous UPEC have been isolated from both females and children with acute cystitis, some of whom suffered from recurrent UTI [69, 84]. In addition, human urothelial cells have supported the development of IBCs in vitro with increased expression of several clinically-relevant virulence factors [85]. Collectively, there is a wealth of evidence supporting the importance of IBCs in UPEC infections and recurrent UTI.

Quiescent Intracellular Reservoirs (QIRs)

The development of quiescent intracellular reservoirs (QIR) are a result of bacterial invasion into the underlying bladder cell layers. QIRs contain small groups [4–10] of UPEC housed within membrane-derived lysosomal-associated membrane protein 1-positive (Lamp-1+) vacuolar compartments [86]. Bacteria within QIRs are non-replicating, chiefly dormant cells that are highly antibiotic resistant, and therefore able to persist in the host despite unfavourable environmental conditions [87–89]. They display little metabolic capacity, are able to maintain membrane potential, and do not undergo obvious morphological differentiation [90, 91]. They are able to avoid stress responses typically encountered in the host urinary tract such as low oxygen, low iron, and low pH, and are therefore able to maintain a viable population under these conditions. During favourable environmental conditions, these bacteria will resume growth and start multiplying; they have been shown to persist for weeks to months and are responsible for many recurring UTIs [90, 92].

Toxins and Nutrient Acquisition

Hemolysin A

Hemolysin A (α-Hly, Hly A) is a 110 kDa prototypical member of the RTX toxin family, a large group of pore-forming exotoxins produced by various Gram-negative bacteria [93]. In *E. coli* it is encoded on the *hlyCABD* operon and is found in 30–60% of cystitis-associated UPEC, 50–75% of UPEC isolated from pyelonephritis infections and <15% of commensal *E. coli* [93–95]. While originally named for its ability to form membrane-bound pores and lyse RBCs, studies over the past several decades have shown it to be a multifunctional protein whose effects are determined largely by its expression levels and timing. Both in vitro experiments and in vivo animal studies have demonstrated that α-Hly induces urothelial cell toxicity and can lead to the death of numerous cell types including macrophages, neutrophils, uroepithelial cells, RBCs and platelets. This killing can be direct or indirect occurring via pore-mediated lysis [94, 96, 97], the induction of apoptosis, pro-inflammatory pyroptosis through NLRP3 inflammasome activation [98, 99] and immune response-mediated tissue destruction. At lower concentrations α-Hly impairs multiple cellular pathways in urothelial cells, macrophages and neutrophils including those associated with cell-cell attachment, cell signaling, metabolism and immune cell recruitment. For example, α-Hly upregulates the expression of several host serine proteases that degrade proteins critical for urothelial cell-cell interactions [100] and the NF-κB pro-inflammatory cascade. One of these proteases is mesotrypsin, which degrades the host cytoskeletal scaffold protein paxillin causing targeted bladder cell exfoliation. α-Hly has also been shown to induce the dephosphorylation of the multifunctional signaling regulator Akt, leading to the blockage of several host metabolic processes and inflammatory signaling pathways [93]. Finally, the toxin is expressed at high levels within IBCs, suggesting its importance during intracellular progression [32]. Overall, the data support tight regulation of α-hemolysin expression by UPEC to balance the optimal levels of host cell exfoliation, immune activity, local inflammation and cell toxicity/death for a successful infection [32].

Cytotoxic Necrotizing Factor

Cytotoxic necrotizing factor (CNF-1) is another exotoxin strongly associated with UPEC infections, with up to 60% of isolates expressing the protein compared to <10% of commensal strains. Its incidence in UPEC is highly dependent upon the presence of α-Hemolysin with studies showing up to 98% of CNF-1 positive isolates are also positive for α-Hly [93, 101]. While its name implies a predominant role in host cell death, studies illustrate that CNF-1 expression chiefly targets the disruption and manipulation of cellular functions in both uroepithelial cells and leukocytes, promoting UPEC colonization and persistence. Upon release by UPEC,

CNF-1 binds to the laminin receptor on host urothelial cells and is taken up via receptor-mediated endocytosis [102]. The toxin deamidates glutamine residues on the Rho, Rac and Cdc42 GTPases constitutively activating them, inducing membrane ruffling and the formation of both filopodia and lamellipodia that result in bacterial envelopment and uptake [103, 104] It also induces multinucleation and stimulates NF-κB activation in urothelial cells which blocks apoptosis of the infected cells and upregulates expression and release of the pro-inflammatory cytokines IL-6, IL-8 and TNF-α. This immune stimulation is not limited to urothelial cells as CNF-1 was recently shown to induce the maturation and activation of monocyte-derived dendritic cells, which release a similar pro-inflammatory cytokine profile alongside driving CD4+ T cell proliferation [105]. Collectively, this causes inflammation and local tissue damage, immune cell recruitment and activation of the adaptive immune response [106]. Further impacting the host is the fact that arriving macrophages and neutrophils are impaired by CNF-1 in their abilities to mediate killing and phagocytosis of infected host cells and UPEC [107–109]. Induced tissue damage alongside diminished immunity help explain CNF-I presence in up to 50% of strains isolated from more severe pyelonephritic infections.

Iron Acquisition

Iron is a critical macronutrient in both eukaryotes and prokaryotes, acting as an enzyme cofactor in numerous cellular processes. During UPEC infection, both host and pathogen employ strategies for its acquisition and sequestration from the other. Host proteins such as transferrin, hemoglobin, ferritin and lactoferrin collectively limit iron accessibility by UPEC and other microorganisms [110]. In response, UPEC produce ferrous iron (Fe^{2+}) receptors, heme receptors and up to four different classes of siderophores, iron-chelating molecules secreted to scavenge available ferric iron (Fe^{3+}) for uptake [111]. Enterobactin is the most common siderophore produced by *E. coli* and numerous other Gram-negative bacteria. Its affinity for iron is higher than those of host proteins, meaning that it can literally "steal" iron away from the host. While this should afford UPEC an advantage during infection, UPEC-induced inflammation and IBC development trigger host urothelial cells and leukocytes to upregulate transferrin receptors and secrete lipocalin 2, a protein able to bind and neutralize iron-bound enterobactin [112]. This host defense is why UPEC isolates typically express up to three additional lipocalin 2-resistant classes of siderophores, namely yersiniabactin, aerobactin and the salmochelins. Yersiniabactin is a phenolate/thiazoline mixed siderophore critical for virulence of several *Yersinia* species and associated with UPEC cystitis and pyelonephritis. It is also able to bind copper which may aid in preventing UPEC phagocytosis by host leukocytes [113]. Aerobactin is a hydroxamate siderophore that can deliver iron directly to intracellular bacterial targets without undergoing hydrolysis, allowing quick recycling for reuse [114]. Salmochelins are a group of glucosylated variants of enterobactin [115, 116] whose receptor may also play a secondary role in promoting UPEC urothelial internalization. All siderophore

receptors require the Ton B cytoplasmic membrane-localized complex for binding and uptake of iron [117], and several have shown promise as potential UPEC vaccine candidates.

Urease

Protein metabolism in humans results in the production of toxic ammonia as a waste byproduct. This ammonia is immediately combined with carbon dioxide to form urea, which is then safely transported to the urinary tract for excretion. Many UPEC isolates as well as numerous uropathogenic strains of *Proteus mirabilis, Staphylococcus saprophyticus*, *Klebsiella* spp., *Pseudomonas* spp., *Corynebacterium* spp. and *Ureaplasma urealyticum* produce urease, a protein that hydrolyzes urea [118]. The enzyme reverts urea back to carbon dioxide and ammonia, providing a valuable source of nitrogen for the microbe. The buildup of ammonia in the urine induces a rapid rise in pH, precipitating numerous ions, proteins and other previously soluble factors into crystals and subsequently bladder/kidney stones. The major stone type associated with urease- producing pathogens is struvite, composed of magnesium ammonium phosphate and calcium carbonate apatite [119]. These stones can form in the bladder, ureters or kidney with the potential to develop into staghorn calculi and even fill the entire collecting system. This can block urinary flow, induce kidney damage and may require surgical intervention. When caused by urease-producing organisms, these calculi are termed "infection stones" as bacteria are buried within the stone, preventing clearance and often inducing more severe pyelonephritic infections. Organisms in the stone are shielded from the effects of host immunity and antimicrobials, and clearance of infection will not occur without complete removal of the stone alongside antimicrobial therapy.

Surviving Antimicrobials

Antimicrobial resistance can be described as genetic changes to a microorganism aimed at altering the antimicrobial itself or its intended target(s) to block activity, thereby allowing survival amidst levels normally able to successfully treat infection. Resistance associated with UTI is on the rise worldwide, leading to increases in recurrent infections, hospitalizations, the development of multi drug resistant strains (MDR – resistant to antimicrobials from at least three different classes simultaneously) and the use of second, third and even fourth line agents for treatment. Indeed, studies conducted over the past 5 years have expressed MDR rates ranging anywhere from 17% to 97% for UPEC isolates globally, with numerous studies showing rates over 50% [120–124]. While resistance can be caused by inherent or acquired mutations specific to an infecting strain or species, a large portion is encoded on genetic elements able to transfer directly between organisms, even across species and genera. Mechanisms of resistance include: β-lactamase expression targeting destruction of penicillin and cephalosporin-class beta lactams (can be

either Narrow [NSBL] or Extended Spectrum [ESBL]) [125–128]; production of enzymes able to alter the active site of aminoglycosides [129], macrolides [130] and chloramphenicol; generation of functional homologous to folate synthesis enzymes that fail to bind sulfamethoxazole and trimethoprim; alteration of DNA gyrase (Gram-negatives) or Type II topoisomerase (Gram-positives) as well as Qnr-mediated protection of these enzymes that block fluoroquinolone activity [131, 132]; surface expression of lipopolysaccharide (Gram negatives) and specific outer membrane proteins that block many hydrophobic agents from entering the cell [133, 134]; and expression of efflux systems that rapidly expel multiple classes of antimicrobials once inside the cell. Since UTI is the one of the most common reasons for prescribing antimicrobial agents worldwide, and low dose prophylaxis in patients with rUTI is commonly employed [135], much of the blame for this resistance is overuse. Recent studies highlight that of the oral agents routinely used in treating UTI, nitrofurantoin is the only one with consistently low resistance rates (typically <10%) across many geographical zones [122, 123, 136–138]. However, poor tissue and bloodstream penetration prevent its use outside of uncomplicated cystitis. Overall, these findings have left physicians with fewer options for successfully treating UTI without conducting wide panel antimicrobial screening of pathogens, prescribing multiple agents simultaneously, considering the current rates of resistance within their geographical area [139] and the unwanted prescribing of third and fourth line agents [140].

Another strategy employed by UTI pathogens to avoid antimicrobial killing is antimicrobial tolerance. This involves mechanisms that promote population survival amidst bactericidal antimicrobial concentrations despite the absence of identifiable resistant cells. Tolerance is observed within biofilms and IBCs as well as in "persister" cells [141], organisms produced at low levels during exponential and stationary phases of growth and at higher levels when the population is subjected to increased stress/danger [142–146]. While it was originally thought that all tolerant cells were completely metabolically dormant, having acquired a quiescent phenotype that failed to take up and/or utilize the antimicrobial agent [147, 148], selective target inactivation has also been shown to occur [149]. In cells displaying this phenotype, metabolic processes targeted by the antimicrobial are inactivated while most others remain intact. This "selective dormancy" provides the cell protection from the inhibitory agent while allowing it to remain metabolically active.

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Urosepsis: Pathogenesis and Treatment

4

Samir Bidnur and Kymora B. Scotland

Clinical Introduction

Urosepsis refers to a severe infection (-sepsis) of the genitourinary system (uro-) and can be secondary to bacterial, fungal or viral sources [1]. However, bacteria are most commonly the cause of this infection. Infections typically begin from retrograde ascent of bacteria from the urethra to the bladder, and eventually include the renal parenchyma. Factors produced from the pathogenic bacteria (including endotoxin, lipopolysaccharides etc.) and the host immune response (driven primarily from the innate immune system) trigger associated downstream physiologic and clinical changes in response to the infection. Sepsis was previously classified according to clinical severity as sepsis, severe sepsis, and septic shock. The accepted conceptualization of sepsis involved an initial reaction to infection termed the systemic inflammatory response syndrome (SIRS) [1, 2]. SIRS was thought to lead to sepsis if not appropriately diagnosed and managed in a timely fashion. This led to the establishment of the Surviving Sepsis campaign, a global initiative aimed at bringing awareness to the importance of speedy treatment of patients with signs of sepsis [3].

Recently, the Third International Consensus Definitions for Sepsis and Septic Shock reclassified the clinical spectrum of sepsis to overcome limitations from the prior definition (for reference included at the end of this chapter) [4, 5]. Sepsis is

S. Bidnur
The Stone Centre at Vancouver General Hospital, Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada
e-mail: sidnur@alumni.ubc.ca

K. B. Scotland (✉)
University of British Columbia, The Stone Centre at Vancouver General Hospital, Jack Bell Research Centre, Vancouver, BC, Canada
e-mail: kymora.scotland@ubc.ca

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Table 4.1 Sequential (sepsis-related) organ failure assessment (SOFA) scoring system for sepsis classification

	Respiratory	Cardiovascular	Hepatic	Coagulation	Renal	Neurologic
Points	PaO_2/FiO_2	MAP	Bilirubin	Platelets	Serum creatinine	GCS
+0	>400	MAP >70 mmHg	<20 μmol/L	Plt > 150 × 10^3/μL	<110 μmol/L	15
+1	<400	MAP <70 mmHg	20–32 μmol/L	Plt < 150150 × 10^3/μL	110–170 μmol/L	13–14
+2	<300	Dopamine <5 μg/kg/min or dobutamine (any dose)	33–101 μmol/L	Plt < 100150 × 10^3/μL	171–299 μmol/L	10–12
+3	<200 AND mechanical ventilation	Dopamine >5 μg/kg/min or (nor) epinephrine <0.1 μg/kg/min	102–204 μmol/L	Plt < 50150 × 10^3/μL	300–440 μmol/L (or UO <500 cc/day)	6–9
+4	<100 AND mechanical ventilation	Dopamine >15 μg/kg/min or (nor) epinephrine >0.1 μg/kg/min	>204 μmol/L	Plt < 20150 × 10^3/μL	>440 μmol/L (or UO <200 cc/day)	<6

Point scores from 0 to 4 are given within 6 domains reflecting body systems compromised in sepsis and septic shock

PaO_2 Partial oxygen tension on arterial blood gas, *FiO_2* Fraction of oxygen administered, *MAP* Mean arterial pressure, *Plt* Platelet, *GCS* Glascow Coma Scale, *UO* Urine output

now defined as "life-threatening organ dysfunction caused by a dysregulated host response to infection (suspected or confirmed) [4]. The new classification system, Sepsis- 3, utilizes the Sequential (sepsis-related) Organ Failure Assessment (SOFA) scoring which is based on six different score groups, one for each vital organ system commonly affected in sepsis – respiratory, cardiovascular, hepatic, coagulation, renal and neurological systems (Table 4.1).

In this system, a SOFA score of ≥2 points is associated with in-hospital mortality of >10% and qualifies the patient as having sepsis [6]. Septic shock in this setting requires the use of vasopressors (Respiratory 3 or 4 points) in addition to serum lactate levels greater than 2 mmol/L following fluid resuscitation, and is associated with a 40% in-hospital mortality [4]. The addition of a serum lactate measure reflects that severe sepsis and septic shock represent profound circulatory, cellular and metabolic abnormalities. The quick SOFA (qSOFA) clinical score was introduced as a more concise bedside point-of-care test, which includes a respiratory rate of >22 breaths per minute, altered mentation, or a

systolic blood pressure of 100 mmHg or less. The recognition of two of these criteria in a patient identifies the patient with sepsis who is at risk of a poor outcome with a 3 to14-fold increase in hospital mortality as compared with a patient whose qSOFA score is under 2 [7].

Sources and Pathogenesis of Urosepsis

The genitourinary tract accounts for 20–30% of sepsis [8]. Sources of urosepsis may include infections of any genitourinary organ: the kidney (i.e. pyelonephritis, pyonephrosis, renal abscess), bladder (i.e. severe cystitis), prostate (i.e. acute bacterial prostatitis, post transrectal ultrasound guided prostate biopsy), testicular or scrotal (i.e. epididymo-orchitis, Fournier's gangrene). Sepsis from obstructive pyelonephritis by urolithiasis is the most common presentation of urosepsis, representing 43% of cases, followed by prostatic etiology in 25%, genitourinary malignancy in 18% and other genitourinary diseases accounting for the remaining 14% [9]. In urosepsis secondary to obstructive pyelonephritis, ureteral stones are the cause in 65% of cases, malignant obstruction in 21%, pregnancy associated obstruction in 5%, anatomic abnormalities in 5% and post urologic procedure in 4% [1]. Bacterial etiology most commonly include: *Escherichia coli*, *Proteus* spp., *Klebsiella* spp., *Enterobacter* spp., *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [1, 10]. In immunocompromised patients *Candida* spp. and *Pseudomonas* spp. should be considered [11].

Bacterial endotoxins, such as lipopolysaccharide (LPS) from the cell wall of gram-negative organisms appear to mediate the systemic manifestation of sepsis (Fig. 4.1). These bacterial components activate the inflammatory, coagulation, and complement systems, stimulating the activity of monocytes, macrophages, neutrophils, and dendritic cells, amongst other inflammatory cell subtypes [12]. LPS-stimulated monocytes play a central role in mediating clinical sepsis, and produce tumour necrosis factor alpha (TNFα) and interleukin (IL)-1 at LPS concentrations of 25–50 pg/mL [13]. In addition to stimulation of inflammatory cells, endotoxin also directly binds receptors in the endothelial cell membrane, which also promotes pro-inflammatory mediators [14].

Early work in this space demonstrated that the rate of release of endotoxin in the blood stream can result in sepsis of different severities. Taudort et al. gave healthy volunteers a 3 ng/kg intravenous bolus of *E. coli* endotoxin versus an infusion over 4 h. The response of inflammatory mediators, specifically TNFα, IL-6 and neutrophil reaction, occurred earlier and was more severe in the bolus group compared to the infusion group [15]. This is directly relevant in the setting of urosepsis secondary to obstruction, as relief of obstruction often results in rapid levels of endotoxemia and thus rapid development of sepsis. The burden of inflammatory mediators in the blood can be prognostic as well. The serum level of TNFα has been shown to correlate with death from urosepsis [16, 17].

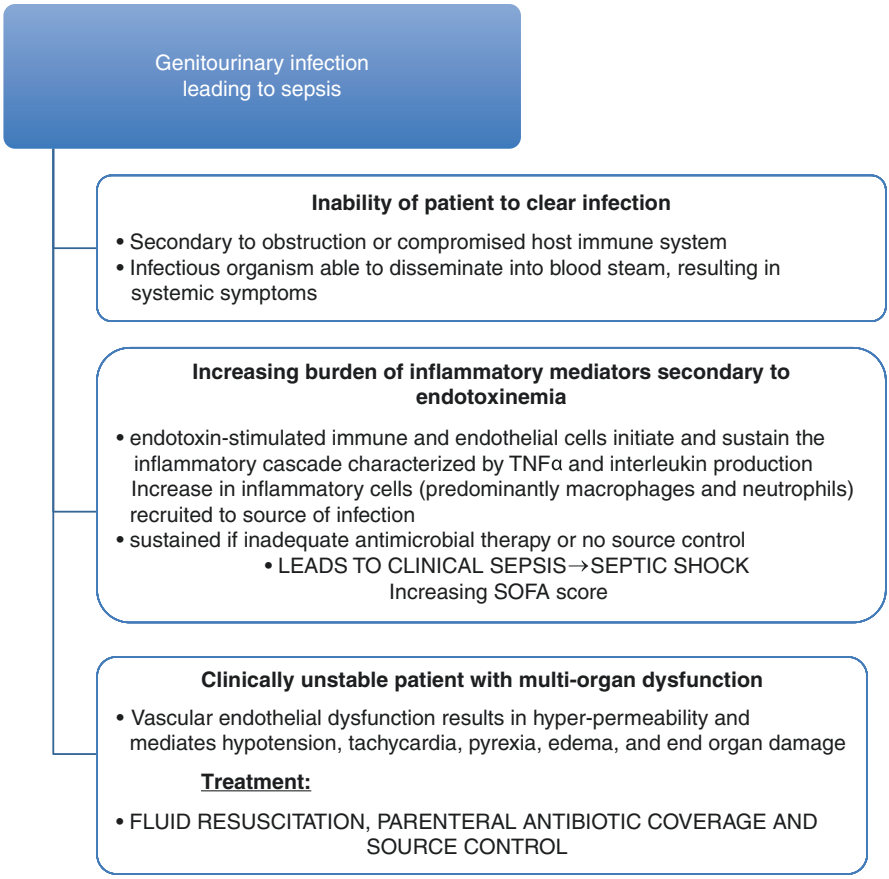

Fig. 4.1 Genitourinary infection leading to sepsis

Management

Managing urosepsis requires prompt recognition, early goal directed resuscitation, broad-spectrum parenteral antibiotics, and source control [18]. Diagnosis is initiated with a focused history which includes assessment of systemic features: fevers, chills, rigors, mental status changes, malaise; urinary symptoms: difficulty voiding, dysuria, gross hematuria or pyuria, flank or abdominal pain, testicular, penile or perineal pain, perineal/scrotal skin changes, recent urologic instrumentation, and urologic history. Physical examination is mandatory, starting with review of vital signs and temperature. Focused exam should assess flank and abdominal tenderness, and palpation of the scrotum and perineum for crepitus. The latter is of paramount importance for the early detection of Fournier's gangrene.

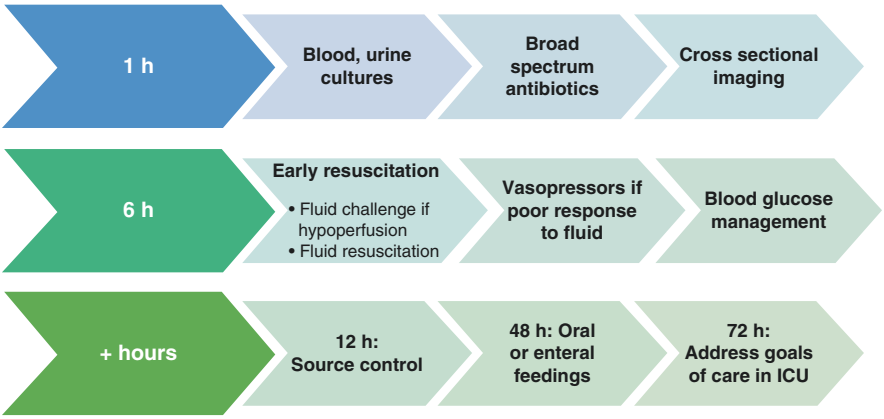

Fig. 4.2 Clinical management of urosepsis

Laboratory investigations should include a complete blood count, electrolytes and renal function tests, serum lactate, urinalysis, blood and urine cultures prior to antibiotic initiation. If history or physical examination identify a potential testicular, scrotal or prostatic source an ultrasound is warranted [1]. If clinical suspicion for a renal etiology, computed tomography (CT) scans are highly sensitive in detecting renal abscesses [19] in addition to hydronephrosis and urolithiasis [20].

Early goal directed therapy are required for reducing mortality and optimizing outcomes as described in the Rivers Protocol [18]. This involves supporting the patient's cardiovascular system with crystalloid fluid resuscitation and vasoactive or inotropic agents if refractory despite euvolemia. The respiratory system is supported with supplemental oxygen and possible mechanical ventilation to maintain tissue and organ oxygenation and perfusion; RBC transfusions are considered to maintain a hematocrit ≥30% to ensure an adequate quantity of circulating RBC's to perfuse tissue and organs. Sedation and paralysis may be considered if the patient is mechanically ventilated to reduce metabolic and oxygen demands in septic shock [18]. Early initiation of empiric parenteral antibiotics, ideally within 1 h of presentation, is essential to minimize mortality [21, 22]. Figure 4.2 provides a general plan for urosepsis management. The authors recommend that physicians search the patient's past medical records for history of a resistant organism. Antibiotic selection should be initially broad to cover bacteria common to urosepsis (see above), and should consider local patterns of resistance and regional antibiograms, patient allergies, and pharmacokinetics and dynamics of urinary tract involvement and tissue penetration [23]. A general antibiotic strategy is to use a third generation cephalosporin combined with enterococcus coverage (i.e. ceftriaxone + ampicillin), or broad-spectrum agents such as piperacillin-tazobactam or a carbapenem, particularly if the local rates of extended spectrum beta lactamase (ESBL) producing organisms is high [24–30]. Once blood and urine cultures have revealed the offending organism and antibiotic sensitivities are available, the antibiotic may be tailored

appropriately. If candiduria or candidemia is present, the addition of antifungal agents is necessary [26, 27].

Source control is paramount and especially important in obstructed systems and in some cases of abscesses. Initially, the goal should be to perform the most minimal procedure necessary to gain adequate drainage or relief of obstruction, with definitive management at a later date once the patient has been clinically stabilized. The classic example is urosepsis secondary to ureteral obstruction from stone disease. The sepsis patient is resuscitated and source control is obtained via nephrostomy drainage or ureteral stenting. Once the patient is stabilized and the urine culture is sterile, the stone may be treated via ureteroscopy or percutaneous nephrolithotomy, often weeks after the occurrence of sepsis. The following are specific recommendations for given clinical scenarios:

- Obstructed pyelonephritis
  - Requires urgent retrograde ureteric stent or nephrostomy tube to decompress an infected system [31, 32].
- Emphysematous Pyelonephritis
  - Consider nephrectomy, or percutaneous drainage if not clinically responding to medical management [33–35].
- Renal or Peri-renal Abscess
  - May consider percutaneous drain if ≥ 3 cm and failure to respond to optimal medical management [36].
- Acute prostatitis
  - If lack of clinical improvement and associated abscess, may consider trans-urethral resection, or trans-rectal drain [37, 38].
- Fournier's Gangrene
  - Requires immediate surgical debridement [39].
  - Can be rapidly progressive, requiring repeat operation to ensure complete debridement

Prior Sepsis Scoring

For reference, the prior sepsis scoring system is detailed here. The severity of sepsis from least to most severe respectively, was classified as:

1. Sepsis
2. Severe Sepsis
3. Septic Shock

Distinguishing among these severities is based upon three sets of criteria. The first criterion is that a clinical infection is suspected or cultured. The second and third criteria are based upon physiologic changes and organ dysfunction respectively.

1. **Criterium 1 Infection Source(1)**
   (a) Clinical suspicion or culture positive infection.
2. **Criterium 2 Systemic Inflammatory Response Syndrome (SIRS)** [1]

(a) Temperature	≤36 °C or ≥38 °C
(b) Heart rate	≥90 beats/min
(c) Respiratory rate	≥20 breaths/min
(d) Respiratory alkalosis	$PaCO_2$ ≤32 mmHg
(e) Leukocytosis	$\leq 4 \times 10^9$/L or $\geq 12 \times 10^9$/L or ≥10% bands

3. **Criterium 3 Multiple Organ Dysfunction Syndrome (MODS)** [1]

(a) Cardiovascular	Systolic blood pressure ≤90 mmHg or mean arterial pressure ≤70 mmHg
(b) Respiratory	PaO_2 ≤ 75 mmHg breathing room air or PaO_2/FiO_2 ≤ 250 if mechanical ventilation
(c) Renal	Urine output ≤0.5 mL/kg post fluid resuscitation
(d) Encephalopathy	Somnolent, agitated, confused, coma
(e) Metabolic acidosis	pH ≤7.3 or base excess ≥5 or lactate ≥1.5 × normal
(f) Thrombocytopenia	≤80 × 10 or ≥50% ↓ in 3 days

Based upon these criteria, the severity of sepsis may be determined [1]:

1. Sepsis – Criterium 1 + ≥2 Criterium 2
2. Severe Sepsis – Criterium 1 + ≥2 Criterium 2 + ≥1 Criterium 3
3. Septic Shock – Criterium 1 + ≥2 Criterium 2 + refractory hypotension ≤90 mmHg

The associated mortality varies with number of criteria involved. Sepsis mortality varies between 7% and 17% if two and four criteria 2 are satisfied respectively; severe sepsis mortality increases by 15–20% for each organ system involved from criterium 3; mortality in septic shock is between 50% and 80% [1].

Conclusion

While urosepsis can result from severe infection of any genitourinary organ, successful clinical management is similar regardless of source: prompt identification of signs and symptoms of sepsis, appropriate intravenous antibiotic administration, and source control. Source control requires decompression of an obstructed system and is pivotal to prevent mortality from urosepsis.

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The Microbiome in the Testis: Epididymitis and Orchitis

5

Benjamin Shiff and Ryan Flannigan

Introduction

Epididymitis and orchitis are by definition inflammation of the epididymis and testes, respectively. While isolated epididymitis is common, orchitis in isolation is quite rare. Rather, orchitis typically occurs in association with epididymitis, a condition referred to as epididymitis-orchitis. It must be noted that not all cases of epididymitis or orchitis develop from a bacterial, or even infectious origin.

This chapter will discuss epididymitis/orchitis in general, but with a focus on the infectious entity.

Epidemiology

Recent epidemiological data on epididymitis and orchitis are lacking, so their incidences in the general population are not well-known. Furthermore, it is difficult to distinguish between epidemiological data for epididymitis and orchitis, as most studies combine these two diagnoses into the general entity of epididymitis-orchitis, even when only one of them is present.

The best data come from the National Hospital Ambulatory Medical Care (NAMC) surveys, which found that epididymitis/orchitis accounted for approximately 0.29% of all visits to office-based physicians (of all specialties), or 1 in 350 [1]. It ranked fifth among genitourinary diagnoses in those aged 18–50 years, behind

B. Shiff
Department of Urology, University of Manitoba, Winnipeg, MB, Canada
e-mail: shiffb@myumanitoba.ca

R. Flannigan (✉)
Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada
e-mail: ryan.flannigan@ubc.ca

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prostatitis, urinary tract infection, renal calculi, and uncomplicated sexually transmitted infections [1]. Overall, there are approximately 600,000 cases per year in the United States [2]. In a Canadian study, epididymitis accounted for 0.9% of visits to a urology practice [3]. Incidence has been shown to be about 25 in 10,000 person-years, and occurs in a bimodal distribution by age, with peaks occurring in men aged 16–30 and 51–70 [4, 5].

Epididymitis

Definition and Classification

Epididymitis by definition is inflammation of the epididymis. Clinically, it can generally be classified into two main entities: acute epididymitis or chronic epididymitis. Most consider a duration of symptoms greater than 6 weeks to differentiate between acute vs. chronic epididymitis, though some studies have utilized a cut-off duration of 3 months [6–8].

Presentation

Acute epididymitis typically presents with a gradual onset of pain and swelling of the epididymis, whereas chronic epididymitis typically presents with pain in the epididymis without swelling, though induration may be present in long-standing cases [2, 6]. Symptoms of a urinary tract infection such as dysuria, frequency, and urgency may be present, as well as fever and chills [2, 6]. It is common for there to be involvement of the ipsilateral testis, indicating epididymitis-orchitis. One study found that involvement of the adjacent testis was present in 58% of cases of epididymitis [5].

Physical examination reveals swelling and tenderness localized to the epididymis, with extension to the adjacent testis in cases of epididymitis-orchitis. Tenderness of the spermatic cord is often present, as well as a reactive inflammatory hydrocele [2, 6]. There may be erythema of the scrotal skin. Classically, relief of pain with elevation of the scrotum was thought to be indicative of epididymitis, a finding known as Prehn's sign [9].

History and physical examination are critical to distinguishing epididymitis from other, more urgent causes of acute scrotum, particularly testicular torsion. Testicular torsion tends to cause sudden onset of pain, and is more likely to be associated with nausea and vomiting. On physical examination, testicular torsion classically manifests as a high-riding testis that may lie transversely within the scrotum rather than its normal vertical lie. An absent cremasteric reflex is also suggestive of testicular torsion [2]. However, these findings are often absent, and clinical judgement is critical in assessment of an acute scrotum. Scrotal ultrasound should be obtained when the clinical picture is unclear and testicular torsion remains high on the differential diagnosis. Torsion of the appendix testis can also present as an acute scrotum. It can present as a blue dot sign which refers to the presence of an area of bluish discolouration over the region of the appendix testis. However, while the blue dot sign has a positive predictive value of 98% according

to one study, its negative predictive value is poor at 35% [10]. A scrotal ultrasound is still recommended to rule out a testicular torsion as the management of torsion of the appendix testis is conservative in nature.

Pathogenesis and Etiology

There are various etiologies for acute epididymitis (Table 5.1) [6]. However, infectious epididymitis is the most common variety, and bacterial epididymitis in particular [11]. The pathogenesis of bacterial epididymitis is retrograde bacterial ascent from the urinary tract [12]. This bacteria may stem from a urinary or sexually transmitted source, and this is strongly correlated with patient age. In sexually active men less than 35 years old, the causative organisms are predominantly *C. trachomatis* and *N. gonorrhoeae*, the typical agents of bacterial urethritis. Conversely, acute epididymitis in pediatric patients and older men is most likely to be caused by coliform bacteria that are typically associated with UTIs. *E. coli* is by far the most common offending organism, accounting for 68% of positive urine cultures in one series of men over 40 years old. This was followed by *Klebsiella* and *Pseudomonas*. In homosexual men who practice anal intercourse, *E. coli* and *Haemophilus influenza* are the most common pathogens causing epididymitis [4, 6, 11, 13].

Tuberculosis has been shown to cause epididymitis, though this is a rare phenomenon. However, it becomes more relevant in endemic regions for this bacterium [6]. BCG treatment for bladder cancer has also been identified as a cause for

Table 5.1 Causes of acute epididymitis

Infectious	Sexually-acquired	Chlamydia trachomatis
		Neisseria gonorrhea
	Urinary infection-related	Escherichia coli
		Proteus spp.
		Klebsiella pneumoniae
		Pseudomonas aeruginosa
	Other infections	**Bacterial**
		Mycobacterium tuberculosis
		Brucella spp.
		Fungal
		Candida albicans
		Histoplasma capsulatum
		Viral
		Mumps virus
		Parasitic
		Schistosoma haematobium
Drug-induced	Amiodarone therapy	
Rheumatic	Behçet disease	
	Henoch-Schoenlein purpura	
Trauma		
Idiopathic		

mycobacterial epididymitis, though there have only been a small number of cases described [14].

Viral, fungal, and parasitic organisms have all been implicated in epididymitis. These are less common however, and more likely in immunosuppressed patients [6].

Rare non-infectious causes of acute epididymitis include amiodarone use and Behçet disease, an inflammatory disorder [15, 16]. A complete history and physical is essential for the identification of these less common etiologies in patients with a negative infectious workup.

Investigations

Laboratory investigations for acute epididymitis should generally include a urethral smear and midstream urine Gram stain and culture and sensitivity. When a urinary pathogen is the cause, the urine Gram stain typically shows a Gram-negative bacillus. Gram negative diplococci on urethral smear are diagnostic of *Neisseria gonorrheae* [6]. Recently, urinary nucleic acid amplification tests for *N. gonorrheae* and *C. trachomatis* have begun to take the place of urethral swabs and have been widely adopted in routine clinical practice [12].

If the diagnosis of epididymitis is unclear in a patient presenting with an acute scrotum, a duplex Doppler scrotal ultrasound can be beneficial, particularly in order to rule out testicular torsion. The primary ultrasound finding for acute epididymitis is increased blood flow to the affected epididymis, in contrast to absent blood flow with testicular torsion. A hydrocele and enlargement of the epididymis may be seen [17]. MRI has also been shown to effectively evaluate an acute scrotum, though given issues of cost and availability, its benefit over ultrasound is questionable [18]. The differential diagnosis of acute scrotum, along with pertinent history, physical exam findings, investigations, and management are reviewed in Table 5.2. Note that not all points in the history, physical examination, and investigations columns listed below need be present in any individual case.

Complications

Acute epididymitis has the potential to cause numerous complications, particularly when treatment is inadequate. These may include epididymal abscess, testicular infarct, atrophy, and subfertility. The suspected mechanism for testicular ischemia in acute epididymitis is inflammatory involvement of the spermatic cord, leading to extrinsic compression of the testicular vessels. Subfertility may result when the testis is involved in cases of epididymitis-orchitis, in which inflammation of the testis results in reduced spermatogenesis [6]. A review of the impact of urogenital infections on fertility found that an episode of acute unilateral bacterial epididymitis is associated with a transient deterioration in semen quality in most patients and permanent damage in a smaller subset of patients [13, 19]. However, bilateral epididymitis predictably results in increased risk of infertility. 30–67% of patients with obstructive azoospermia are due to obstruction at the epididymis, and of these, 20–30% of cases are due to previous infection [20, 21]. *Gonorrhea*, *Mycoplasma*, and *Chlamydia* species were found to be most associated with infectious-related obstructive

Table 5.2 Differential diagnosis of acute scrotum

	History	Physical	Investigations	Management
Epididymitis and/or orchitis	Gradual pain onset	Tenderness of the epididymis and/or testis	Positive urine or STI testing	Antibiotics
		Possible fever		
		Positive Prehn's sign	Increased blood flow on ultrasound	Anti-inflammatories
	Urinary symptoms	Present cremasteric reflex		Scrotal support, heat/cold application
	STI risk factors			
Testicular torsion	Sudden pain onset	Negative Prehn's sign	Negative urine and STI testing	Manual detorsion
	Associated nausea/vomiting	Negative cremasteric reflex	No testicular blood flow on ultrasound	Surgical exploration, detorsion, orchiopexy
	Afebrile	Transverse testicular lie		
	No urinary symptoms	High-riding testis		
Torsion of the appendix testis	Subacute pain onset	Tenderness localized to superior aspect of testis	Ultrasound shows a torsed appendage with normal testicular blood flow	Anti-inflammatories
		Blue dot sign		Scrotal support, heat/cold application
Incarcerated/strangulated inguinal hernia	Bowel obstruction symptoms (nausea/vomiting)	Audible bowel sounds within scrotum	Bowel within the scrotum on ultrasound	Surgical repair
Fournier's gangrene	Risk factors: immunosuppression (chemotherapy, HIV), diabetes, etc.	Diffuse tenderness, out of proportion to visible exam	Ultrasound may show gas in the subcutaneous tissues.	Aggressive resection/debridement
	History of pain and swelling of the perineum/scotum/penis	Rapidly progressing skin changes.		Broad-spectrum IV antibiotics
		Febrile, toxic-appearing		Supportive measures
		Crepitus		
		Cutaneous anesthesia		
		Foul odour		
Testicular rupture	History of trauma	Testicular tenderness	Heterogeneous testicular echotexture on ultrasound	Scrotal exploration + testicular repair or orchiectomy
	Sudden pain onset	Scrotal hematoma	Disruption of tunica albuginea on ultrasound (only seen in 20% of cases). Extruding seminiferous tubules may also be visible.	
Testicular tumor	Classically a painless mass, though less commonly there may be gradual pain onset.	Firm testicular mass	Well-defined hypoechoic mass on ultrasound (for seminomas)	Radical inguinal orchiectomy
		5–10% have an associated hydrocele	Serum tumor markers (AFP, LDH, beta-hCG)	

azoospermia in a recent Chinese study [22]. These infections cause inflammation and scarring of the epididymis and vas deferens, leading to obstruction. Finally, chronic epididymitis is an additional potential complication of acute epididymitis [6].

Management

Proper management of acute infectious epididymitis depends on the likely pathogen responsible. According to the 2015 update of the Center for Disease Control and Prevention guideline for epididymitis, cases suspected to be caused by sexually transmitted *Chlamydia* or *gonorrhea* should be treated with ceftriaxone 250 mg in a single intramuscular dose, as well as doxycycline 100 mg orally twice a day for 10 days. If an enteric organism is the suspected cause, the guideline recommends levofloxacin 500 mg orally daily for 10 days, or ofloxacin 300 mg twice daily for 10 days [23]. These treatment guidelines have been proven effective despite increasing rates of fluoroquinolone-resistant bacteria in much of the world [11]. However, as geographical patterns of bacterial sensitivity and resistance exist, it is essential for the clinician to review their local antibiograms and determine local sensitivity patterns to suspected pathogens. Furthermore, when available, culture directed antibiotic choices should be pursued. Patients with chronic infectious epididymitis should be treated with 4–6 weeks of antibiotics [24]. All patients with acute epididymitis may also benefit from more conservative measures such as anti-inflammatory agents, analgesics, and scrotal support [6].

Orchitis

Epidemiology

As previously discussed, the majority of orchitis cases occur concomitantly with epididymitis, and are therefore more accurately referred to as epididymo-orchitis. The epidemiology of epididymitis-orchitis has already been examined. Isolated orchitis is much less common, and is typically the result of mumps infection. Prior to the introduction of the mumps vaccine to the United States in 1967, approximately 186,000 cases were reported each year. This number has since decreased by over 99%, with a yearly incidence fluctuating between several hundred and a few thousand cases [25]. However, the incidence of mumps has increased in the past 3 years, with over 6000 cases reported in 2016 and 2017, up from 229 cases in 2012 [25]. Orchitis is the most common complication of mumps in post-pubertal men, occurring is 20–30% of mumps cases. Among cases of mumps orchitis, 20–30% occur bilaterally [26].

Definition and Classification

Orchitis by definition is inflammation of the testicle. Similar to epididymitis, it can clinically be classified into two main entities: acute orchitis or chronic orchitis. As with epididymitis, most consider a duration of symptoms greater than 6 weeks to define acute vs. chronic orchitis, though some studies have utilized a cut-off duration of 3 months [7, 8].

Presentation

As orchitis is most often associated with epididymitis, its presentation tends to be very similar to that of epididymitis which has been described. Indeed, the swelling associated with presentations of acute scrotum often make it difficult to differentiate between the epididymis and the testis, so distinguishing between epididymitis, orchitis, or epididymitis-orchitis can be a challenge. In cases of true isolated orchitis, tenderness restricted to the testicle itself may be elicited. As isolated orchitis is typically a complication of mumps infection, this presentation is often accompanied with other features of this viral illness, such as parotitis, malaise, and myalgias [10, 26]. On history for possible mumps orchitis, it is important to assess immunization status and possible exposures to infected individuals. Importantly, mumps infections may arise in previously immunized individuals, though unimmunized patients are of course at highest risk.

Pathogenesis and Etiology

Most cases of orchitis are associated with ipsilateral epididymitis, and occur because of local spread of infection from the epididymis. Therefore, the etiology of orchitis in these instances is identical to that of bacterial epididymitis that has been previously discussed. However, isolated orchitis is typically caused by hematogenous seeding of a viral infection, most commonly mumps.

Investigations

Patients with isolated orchitis should undergo testing for mumps infection. This includes buccal or oral swab testing, as well as serology [25]. Furthermore, as it is often difficult to distinguish between isolated orchitis and epididymo-orchitis, these patients should undergo the usual tests for urinary or sexually transmitted infection, despite the fact that these are rarely associated with isolated orchitis.

Complications

30–50% of patients with mumps orchitis will develop testicular atrophy [26]. The virus infiltrates the testicular parenchyma, leading to parenchymal inflammation with recruitment of lymphocytes into the testicular interstitium. The inelastic tunica albuginea surrounding the testicle creates a rigid barrier, and therefore any swelling results in a rise in intratesticular pressure and subsequent pressure-induced testicular atrophy [26]. Mumps orchitis rarely leads to sterility, but it may contribute to impaired fertility. Subfertility is estimated to occur in 13% of patients with unilateral mumps orchitis, while up to 87% of patients with bilateral mumps orchitis may experience infertility [26, 27].

Management

General treatment principles of acute orchitis are similar to those for acute epididymitis. Antibiotic therapy should be utilized in cases where a bacterial etiology is suspected, and antibiotic choice should ideally be based on culture and sensitivity testing, or at minimum directed towards the most likely causative organism. General conservative measures are once again important, such as rest, anti-inflammatory agents, intermittent icing and scrotal support. There are no specific

anti-viral therapies for mumps orchitis, so the usual conservative measures are especially important in these cases. Steroids may help in diminishing the pain and edema, but they do not alter the clinical course of the disease or prevent future complications [26]. There is a some evidence that interferon, by inhibiting transcriptase-induced viral replication, could inhibit the development of testicular atrophy [28, 29]. However, this is not standard therapy at this time and remains under investigation.

Chronic Scrotal Pain

Men presenting with chronic epididymal or scrotal pain have long been given the diagnosis of chronic epididymitis/orchitis or epididymyalgia/orchalgia. However, with recent years has come the acknowledgement that these patients often have no identifiable infectious or inflammatory process at work. Rather, there is often no clear etiology for these conditions, and there has been somewhat of a paradigm shift towards considering these entities as a chronic pain condition. The encompassing term *chronic scrotal pain* reflects this paradigm, and is defined as pain localized to the scrotal structures (testis, epididymis, spermatic cord) that has a duration of over 3 months and significantly interferes with daily activities [30]. The etiology of this condition is not well understood. Post-vasectomy chronic scrotal pain is a well-documented phenomenon. Other etiologies such as recurrent infections, trauma, and scrotal conditions such as hydrocele, spermatocele, and varicocele may all contribute to the development of chronic scrotal pain [31].

The full armament of conservative measures should be considered in the management of this difficult condition. These include medications such as NSAIDs, analgesics, and neuropathic pain medications. A trial of 4–6 weeks of antibiotics should be given if there is suspicion of true chronic infectious epididymitis or orchitis. Non-pharmacologic conservative measures such as physiotherapy, acupuncture, and psychotherapy may also provide benefit. Surgical intervention in the form of epididymectomy or orchiectomy should be considered only when all conservative measures have failed. Alternatively, microsurgical denervation of the spermatic cord has been shown to be effective, and may be a valid substitute for more radical surgery [32]. The only surgical intervention with sufficient evidence for its recommended use is vasovasostomy for the relief of post-vasectomy chronic pain. Additional strategies under investigation include Botox injections and delayed release analgesics [33].

Testis Microbiome: Future Directions of Research

While extensive research has been conducted investigating the microbiome of the gut and how it influences physiologic process both locally and distant within the body, the microbiome of the human testis is largely unknown. A recent study has identified different testis biomes among fertile and infertile men, suggesting an association between the testis biome and spermatogenesis. Specifically, the testis microbiome within men with normal spermatogenesis was characterized by the presence of

Actibobacteria, *Bacteroidetes*, *Firmcutes*, and *Proteobacteria* as the dominating phyla. In contrast, men with idiopathic non-obstructive azoospermia were found to have increased amounts of bacterial DNA present with associated reduction in bacterial diversity, mainly due to lack of *Bacteroides* and *Proteobacteria* [34].

Epididymitis-related infertility is mainly the result of obstructive azoospermia, in contrast to the patients assessed in this study. However, the testis microbiome may play an important role in the subfertility occasionally associated with infection extending to the testicle, in which spermatogenesis has been shown to be reduced. The previously mentioned study was the first to examine the relationship between the testis microbiome and fertility, but further exploration into this area will surely be forthcoming.

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Struvite Stone Formation by Ureolytic Biofilms

6

Erika J. Espinosa-Ortiz and Robin Gerlach

Formation of Struvite Stones by Ureolytic Microorganisms

Infection stones account for almost 10–15% of urinary tract stones in the general population. Infection stone formation is one of the most complicated stone diseases due to the association of stone growth with bacterial infections, fast stone growth, high recurrence and tendency to form large aggregates (e.g. staghorn calculi) [1]. Infection stones are comprised of struvite ($MgNH_4PO_4 \cdot 6H_2O$), which is frequently accompanied by calcium phosphate, mostly in the form of carbonate apatite (carbapatite, $Ca_{10}(PO_4)_6CO_3$). Indeed, the composition of -so called- struvite stones is quite heterogeneous; Flannigan et al. [2] reported that among 121 struvite stones collected, 13.2% were homogeneous struvite, whereas 86.8% were heterogenous stones admixed with calcium phosphate (42.1%), calcium oxalate (33.9%), calcium carbonate (27.3%), and uric acid (5.8%).

Formation of stones is an intricate process that starts with urine ***supersaturation*** (presence of a solute in solution at a higher concentration than that of its own solubility), leading to crystal ***nucleation*** and proceeds via ***crystal growth*** along with the ***aggregation*** of crystals [3]. Supersaturation in urine is a prerequisite for stone formation, however supersaturated urine is also observed in non-stone formers. Supersaturation implies that mineral precipitation is possible, but the presence of inhibitors (e.g. proteins, polysaccharides, etc.) [4] and slow kinetics of precipitation can keep urine supersaturated without significant mineral precipitation or stone growth. In the urinary tract, nucleation of crystals most likely occurs on pre-existing surfaces (heterogeneous nucleation); crystals usually become associated with

E. J. Espinosa-Ortiz · R. Gerlach (✉)
Department of Chemical and Biological Engineering, Center for Biofilm Engineering, Montana State University, Bozeman, MT, USA
e-mail: erika.espinosaortiz@montana.edu; robin_g@montana.edu

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surfaces ranging from suspended particles in urine (e.g. bacteria, organic substances, cellular debris), to the urothelium, to medical devices (e.g. ureter, catheter) [5]. Formation of microcrystals (roughly those ≤20 μm) is also observed in non-stone formers, but the crystals are small enough to be excreted through the urinary tract. Thus, for a stone to form, crystals do not only need to be formed but also need to grow and aggregate to be retained within the urinary tract.

The formation of infection stones depends on urine chemistry, which is driven by two key processes [1]: (i) urine pH; a pH >7.2 is commonly considered the threshold for struvite precipitation, and (ii) supersaturation of urine with respect to the solubility product of magnesium (Mg^{2+}), ammonium (NH_4^+) and phosphate (PO_4^{3-}). Urine normally contains Ca^{2+}, Mg^{2+} and PO_4^{3-}, but the concentration of NH_4^+ in urine is usually not high enough to promote struvite precipitation. Formation of infection stones seems to be associated with ***urinary tract infections*** (UTIs) [1], particularly with the presence of ***ureolytic*** (urea-hydrolyzing) microorganisms. Ureolytic microorganisms produce the enzyme urease [6], which catalyzes the hydrolysis of urea ($CO(NH_2)_2$) (also known as ureolysis) that leads to the production of ammonia (NH_3) and carbon dioxide (CO_2) (Fig. 6.1, Eq. 1). NH_3 functions as a Bronsted-Lowry base, generating hydroxide (OH^-) ions, resulting in urine alkalization and production of NH_4^+ (Fig. 6.1, Eq. 2), and ultimately triggering struvite precipitation (Fig. 6.1, Eq. 7). CO_2 generation during ureolysis leads to the production of carbonate (CO_3^{2-}) and bicarbonate (HCO_3^-) ions (Fig. 6.1, Eqs. 3–5). Formation of CO_3^{2-} can promote the precipitation of carbapatite and calcium carbonate (Fig. 6.1, Eqs. 6 and 8), which have been described to occur at pH 6.8 and lower. Carbapatite crystals appear to aggregate as the pH increases during ureolysis [7].

The particular combination of elevated NH_4^+ concentration and alkaline urine pH promoting struvite precipitation is almost exclusively associated with the infection

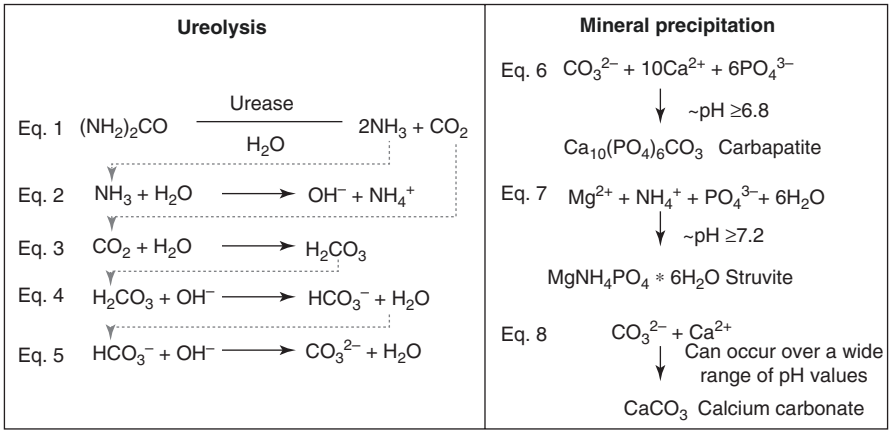

Fig. 6.1 Chemical reactions involved in the hydrolysis of urea and the resulting mineral precipitation induced by ureolytic microorganisms

of the urinary tract with ureolytic microorganisms, most commonly *Proteus* spp., *Klebsiella* spp., *Pseudomonas* spp., *Staphylococcus* spp. and some species of *E. coli* [1, 8, 9]. *P. mirabilis*, a frequent microorganism involved in UTIs [10], has been the most commonly detected microorganism to be associated with struvite formation.

Biofilm Development and Struvite Precipitation

The presence of pathogens in the urinary tract can lead to ***biofilm*** formation, i.e. surface-attached microbial communities embedded in self-produced extracellular polymeric substances (EPS) [11, 12]. Biofilms are generally more resistant to physical and chemical stresses compared to their planktonic counterparts [11, 13, 14]. Biofilm resistance is attributed to the protective effect of the EPS matrix that can hinder the transport of antimicrobials into the biofilm, to the formation of chemical and metabolic gradients within biofilms, and due to other possible resistance mechanisms [13, 15].

Ureolytic biofilm formation in the urinary tract may play a key role in struvite stone formation, as ureolytic microorganisms can provide the appropriate conditions for struvite precipitation, and the EPS matrix can act as additional nucleation site for crystal development. Biofilm development and mineral precipitation in the urinary tract seem to follow a series of steps [16]. ***(i) attachment of ureolytic microorganisms in the urinary tract***: a thin layer is formed comprised of planktonic cells and urinary metabolites, and ureolysis possibly commences; ***(ii) formation of microcolonies***: this is often the first phase of biofilm development accompanied by production of EPS; ureolysis increases the pH, NH_4^+ and CO_3^{2-} concentrations in the urine; ***(iii) formation of crystals***: primary crystals start forming due to alkaline conditions and increase of NH_4^+ and CO_3^{2-} in urine (supersaturation leads to carbapatite, struvite and possibly calcium carbonate precipitation, Eqs. 6–8); crystals can become entrapped in the EPS matrix; detachment of microbes from the biofilm may occur; ***(iv)*** **crystal growth/aggregation and secondary nucleation**: crystals form and start growing around the attached bacteria and possibly outside the EPS matrix; planktonic bacteria adhere to pre-existing crystals resulting in the formation of more microcolonies that also precipitate minerals; this results in the formation of layers of bacteria encased in minerals; crystal aggregation is influenced by organic macromolecules in urine (e.g. proteins, lipids, glycosaminoglycans), which can inhibit or promote crystal aggregation [17]; ***(v)*** **stone formation** is the result of **repeated cycles of the previous steps** (bacterial attachment, formation of microcolonies/biofilms and growth/aggregation of crystals) to integrate minerals within the bacterial biofilm, and vice versa.

The inherent heterogeneous structural, chemical and biological nature of biofilms [13] influences the biomineralization process taking place in the urinary tract (Fig. 6.2). Urine flow and chemistry vary spatially in the urinary tract; ureolytic microorganisms hydrolyze urea, generating NH_4^+, changing the local urine saturation; the natural heterogeneity of biofilms [13] creates gradients of NH_4^+ concentration, and induces mineral precipitation within the biofilm (Fig. 6.2). Urea concentration

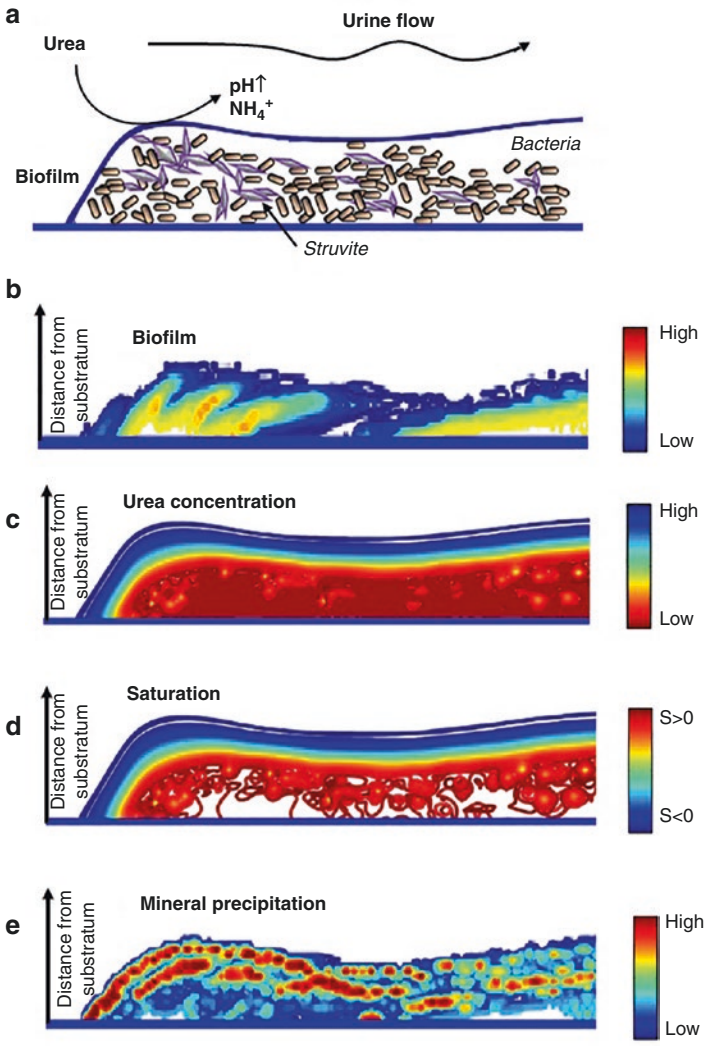

Fig. 6.2 Biofilm induced struvite precipitation. (**a**) Ureolytic biofilm cross-section in the urinary tract with continuous urine external flow. Ureolytic microorganisms hydrolyze urea, which results in an increase of pH values as well as ammonium (NH_4^+) and carbonate (CO_3^{2-}) concentrations in the biofilm and the urine. Minerals form and become entrapped inside and around the microbial biofilm. (**b**) Shear stress from the urine flow can stretch and deform the biofilm. (**c**) Transport of urea within the biofilm occurs via diffusion; ureolysis within the biofilm decreases urea concentration inside the biofilms and induces heterogeneity. (**d**) Certain areas within and around the biofilm become oversaturated with respect to struvite due to the production of NH_4^+ (saturation index, $S > 0$); as the biofilm grows thicker areas of supersaturated environment increase. (**e**) As a result of oversaturation within the biofilm, it is more likely for minerals to precipitate. Mineral precipitation can occur rapidly at the biofilm-urine interface, as the external flow of urine efficiently transports ions to the biofilm surface. This figure was modified based on the results from a mathematical model of biofilm-induced calcite precipitation developed by Zhang and Klapper [18]. (Figured modified and reproduced from Zhang and Klapper [18], with permission from the copyright holders, IWA Publishing)

heterogeneities arise in biofilms due to reaction-diffusion interactions; urea concentration decreases with depth into the biofilm as ureolysis occurs. Oversaturation with respect to minerals such as struvite occurs within and around biofilms owing to the production of NH_4^+, carbonates, hydroxyl ions, etc. to increase the saturation index to greater than 0. As the biofilm grows thicker more highly saturated zones develop, which increase the likelihood of mineral precipitation in and around biofilms. Mineral precipitation can potentially occur rapidly at the biofilm-urine interface, as the external flow of urine efficiently transports ions to the biofilm [18].

Investigation of Struvite Stone Formation Using In Vitro and In Silico Techniques

As outlined, infection stone formation is an intricate multi-step process influenced by physicochemical, biochemical and physiological factors. Due to the complexity of biofilm development combined with mineral precipitation in the urinary tract, **in vivo** observations of stone formation have been hitherto very challenging. The use of controlled laboratory experiments (**in vitro**) along with computer simulations (**in silico**) is an attractive alternative to investigating and ultimately developing strategies to control stone formation. In vitro and in silico experiments indeed allow for the study of microbe-mineral interactions at the required spatial and temporal resolution relevant to stone formation. Understanding key microbe-mineral interactions is anticipated to be a key in the development of new treatments and management strategies for struvite stones.

Controlled laboratory experiments such as batch and continuous flow systems have been used to investigate struvite stone formation and the effect of different urine conditions on stone development. Batch experiments have been performed to evaluate (i) the effect of inhibitor or promoter substances on struvite crystallization [7, 19, 20], (ii) the effect of urease inhibitors [21], (iii) the influence of substances potentially inhibiting microbial colonization and biofilm development by uropathogens [22–24], and (iv) the resistance of materials to encrustation [25]. However, batch experiments generally do not simulate the flow conditions present in the urinary tract, and generally do not allow for long-term experimentation and assessments. Continuous flow systems allow to simulate the hydraulic conditions in the urinary tract and -if designed properly- for real-time observations over extended periods of time. Flow systems have been mostly used to test ureteral stents, antimicrobials and precipitation-inhibitors (both compounds and devices/materials) [26, 27]. Due to demonstrated reproducibility of results, the Center for Disease Control (CDC) biofilm reactor [28] has been suggested to be used for the evaluation of urological device materials [26]. The CDC reactor allows for the observation of biofilm growth and mineral precipitation over time under tightly controlled conditions using removable coupons (Fig. 6.3a). Flow systems have also been developed to test the resistance of different biomaterials used in urinary tract devices against colonization and encrustation (e.g. silicone, polyurethane, Percuflex) [27]; flow systems allow for the

exposure of stent devices to flows similar to those in the urinary tract (Fig. 6.3b). Hobbs et al. [29] developed an in vitro laboratory model to investigate infection stone formation. The model simulates the urinary tract system using a series of analog components: CDC reactors as kidney and bladder analogues, tubing and glass capillaries simulating the ureter, and sponge coupons representing the porous structure of the kidneys. This system indeed allows for the real-time observation of biofilm formation and mineral precipitation (Fig. 6.3c). The CDC reactor used as kidney analog allows for sampling of the coupons over time; biofilm-mineral composites accumulate on the coupons as a result of ureolysis induced by ureolytic microorganisms in the reactor; glass capillaries integrated in the "ureter" line allow for microscopic observations over time to assess bacterial migration or mineral precipitation.

Investigation of biofilm formation and mineral precipitation in the urinary tract should consider the effect of physicochemical factors including ***reaction*** (e.g. ureolysis and precipitation), ***transport*** (e.g. advection and diffusion), and ***hydrodynamics*** (e.g. fluid flow and shear stress). Furthermore, the viscoelasticity and mechanical properties of the biofilm-mineral composites should be considered when investigating mineral precipitation in infected urinary tract systems. As stated above, biofilms are non-uniform systems with structural, biological and chemical heterogeneity; moreover, biofilms in the urinary tract are subjected to fluctuating conditions, such as variations in urine flow and urine chemistry [18]. Quantitative descriptions of urine flow and urine chemistry can be obtained using mathematical modeling along with the simulation of biofilm development and mineral precipitation similarly to work done in the development of engineered biomineralization technologies [30–35].

Reactive transport modeling is a tool commonly used in engineering that allows for the analysis of coupled physical, chemical and biological processes [36]. An example of a user-friendly modeling program that can be used to model reactive transport is COMSOL Multiphysics® software (a finite element framework, COMSOL Inc., Burlington, MA, USA). Connolly et al. [33] used this software to estimate biofilm-specific kinetics of ureolytic biofilms (*E. coli* MJK2) in flow systems. Estimating ureolysis biofilm-kinetics along with other tools to characterize biofilms (e.g. confocal microscopy) can aid in understanding local chemical gradients in ureolytic biofilms. Hence, determining biofilm-specific kinetics can provide a more accurate representation of biofilms in flow systems, for example, development of ureolytic biofilms on urethral catheters [37].

Geochemical modeling software can simulate the saturation conditions of aqueous solutions (e.g. urine) under a variety of conditions and can predict the likelihood of precipitation. Geochemical modeling estimates the saturation state of a system by considering the chemical species in solution and the influence of solid phases. Common geochemical modeling packages are Visual MINTEQ [38] and PHREEQC [39]. These computer-based models can be used to simulate saturation conditions, pH and ionic strength in urine, and thus predict the potential for mineral (e.g. struvite) precipitation [40], which could lead to stone formation. Combinations of different modeling tools (e.g. COMSOL-PHREEQC) [41] can provide an insight into

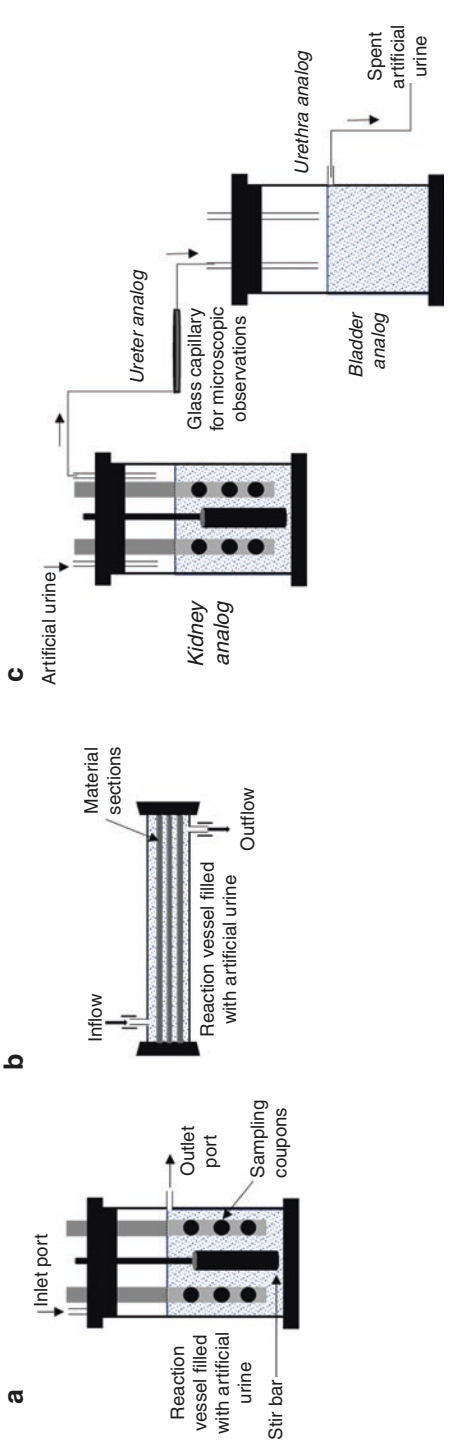

Fig. 6.3 Common continuous flow systems used to study stone formation and to test urological device materials. (**a**) CDC reactor. (**b**) Dynamic encrustation system. (**c**) Laboratory model mimicking urinary tract system

systems as complex as the urinary tract, in which formation of ureolytic biofilms under flow conditions along with mineral precipitation is occurring, taking into account urine chemistry and changes in hydrodynamics.

Summary

The formation of struvite stones ($MgNH_4PO_4 \cdot 6H_2O$), also known as infection stones, is typically associated with urinary tract infections, particularly, with ureolytic microorganisms. These microorganisms hydrolyze urea, produce ammonium (NH_4^+) and potentially increase the urine pH. This process, in turn, can affect urine chemistry and can promote struvite and other mineral precipitation. Ureolytic biofilm development in the urinary tract (e.g. on implanted devices) can provide the conditions necessary for struvite precipitation and stone formation. Factors that influence stone formation and that need to be considered when investigating infection stone formation include: (i) the structural, chemical and biological heterogeneity of biofilms, (ii) urine chemistry and its effect on mineral precipitation and biofilm formation; (iii) presence of modulators in urine (inhibitors or promoters of crystal growth and aggregation, e.g. organics), and (iv) the hydrodynamic conditions (flow) in the urinary tract. Controlled experiments and computer simulations can aid in better understanding the microbe-fluid-mineral interactions occurring within ureolytic biofilms in the urinary tract system. A more comprehensive understanding of stone formation is predicted to ultimately lead to the development of improved management and prevention strategies for infection stones.

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Management of Infection Stones

7

Tim Large and Amy E. Krambeck

Introduction

Infections stones are an amalgam of stone compositions and stone structures. Bacterial induced pH perturbations create the necessary supraphysiologic alkali environment for such stones to develop. Infection stones are estimated to account for 10–15% of all stones and are up to three times more common in women than men [1]. Struvite infection stones are a composite of magnesium ammonium phosphate crystals (also known as triple phosphate stones) and represent a subset of infectious stones (Fig. 7.1) [1]. Calcium carbonate apatite (also known as carbonate apatite) is another stone type with a strong association (64% of cases) to bacterial infections; however, the authors reporting on this association explained that all stone compositions can harbor infections [2]. There are many bacteria with the capacity to convert urea into ammonia and carbon dioxide [3]. They have been identified as urea-splitters, some of which include gram-negative genera such as *Proteus, Klebsiella, Pseudomonas, Providencia, Morganella* and the gram-positive genus *Staphylococcus* [3]. Interestingly, *Escherichia coli* is the most common genitourinary pathogen and has been isolated from infection stones despite traditionally being thought of as a non-urea splitter. It is somewhat controversial as to whether or not *E. coli* is a facultative urea-splitter. Standard metabolic stones (calcium based stones) can also be secondarily infected with bacteria leading to recurrent urinary tract infections (UTI); however in such cases the stone is present

T. Large
Indiana University, Indianapolis, IN, USA
e-mail: timlarge@iupui.edu

A. E. Krambeck (✉)
Indiana University/IU Health Physicians, Indianapolis, IN, USA
e-mail: akrambeck@iuhealth.org

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Fig. 7.1 Example of a staghorn struvite stone isolated from a robotic pyelolithotomy

before the infection occurs and is not the result of the infection [4]. True infection stone formation can occur rapidly (4–6 weeks), is more common in larger stones (>2 cm), and accounts for approximately 50% of staghorn calculi [5]. Nonetheless, the clinical presentation of a patient, rather than the pathophysiology yielding their stone drives the initial management of a patient afflicted with an infection stone.

The majority of patients with infection stones present with vague abdominal or flank pain, recurrent UTIs, or hematuria. In almost 10% of cases the patient is completely asymptomatic; however, treatment with antibiotics, especially in the setting of infections or sepsis (rare ≤1%), is an appropriate starting point to stabilize the patient [6]. Empiric oral antibiotics such as combination penicillins, fluoroquinolones or third generation cephalosporins can be used until urinary bacterial profiling with antibiotic sensitivities is achieved, and targeted antibiotic therapy is initiated [7]. Decompression of the collecting system with a ureteral stent or nephrostomy tube may be necessary if there is evidence of obstruction on clinical history and/or imaging studies [8]. Prior to intervention, patients with infection stones should undergo a full medical evaluation, including pertinent genitourinary history like urinary dysfunction (retention, neurogenic bladder) or reconstruction. Basic laboratory tests including a urinalysis, a basic metabolic panel (renal insufficiency, hyperkalemia), a complete blood count are essential before initiating any stone therapy. Lastly, imaging (non-contracted CT, abdominal x-ray, ultrasound) is necessary to characterize the size, location, and number of an infectious stone as well as the presence or absence of hydronephrosis [8].

Subsequent management of the stabilized or asymptomatic patient is determined by their health status. The most current guidelines published by the European and American Urologic Societies recommend that any patient deemed appropriate for surgery should undergo surgical stone removal. Percutaneous nephrolithotomy is recommended in almost all cases; however in certain rare instances, such as ectopic kidney, a laparoscopic pyelolithotomy or anatrophic nephrolithtomy is an acceptable alternative [9]. In general ureteroscopic stone removal is not recommended due to the high rate of residual calculi and that most infection stones are generally large. The recommendation to surgically remove all stones stems from early data suggesting that higher rates of renal failure (36% vs 15%) [10] and mortality (28% vs 7.2%) [11] occur in patients with infection staghorn stones after 7–10 years of conservative therapy versus surgery. A recent publication from the United Kingdom following 22 patients for approximately 8 year attempted to reconstitute conservative management as an option for asymptomatic patients with staghorn stones. The authors found that progressive renal failure and all-cause mortality occurred in 6% vs 40% and 29% vs 60% of patients with unilateral versus bilateral infectious staghorn stones. They concluded that the conservative management of staghorn calculi in surgically unfit patients is safer than previous studies would suggest. However, their ideal patient to observe did not include patients with bilateral infections stones as 80% of these individuals had at least 1 UTI or hospital admission and 40% of the patients died as a result of their stones.

Conservative management of patients with infectious stones includes observation, dietary counseling, urine acidification, antibiotic therapy and urease inhibitors [12]. Dietary modifications have largely been supplanted by surgical therapies; however, in the 1940s, Dr. Shorr successfully showed stone dissolution and reduction in stone growth with dietary modifications [13]. In addition to a low-phosphate and low-calcium diet, oral estrogens along with aluminum hydroxide gel were used to reduce calcium and phosphate excretion in the urine. Unfortunately, the diet was poorly tolerated and largely abandoned as therapy for infection stones [14]. Urine acidification and stone dissolution therapy has shared a similar fate in its application for the treatment of infectious stones. Original studies used boric acid, permanganate, renacidin R and solution G and showed successful stone dissolution in up 80% of cases. Despite its success, dissolution therapy is rarely utilized because of the associated risks. Dissolution therapy has been associated with hypermagnesemia with resulting seizures, sepsis and even death. Furthermore, the therapy is costly, requiring prolonged hospital stays for monitoring and percutaneous instillation of solvent through a nephrostomy tube [13, 15]. Oral urease inhibitors offered great potential with several randomized controlled trials showing inhibition of stone growth in 93–100% of patients taking acetohydroxamic acid (AHA), compared to 37–46% in the placebo arm [16]. Unfortunately, the side effect profile of AHA, especially those affecting the psychoneurologic and musculo-integumentary symptoms, resulted in more than 50% of patients stopping therapy. Thus, the significant side effect profile of AHA vastly limits its clinical applicability [17]. Despite there being a variety of conservative approaches to managing patients with infectious stones, due to side effects and potential complications these should be regarded as palliative for patients unable to undergo surgical intervention.

There are multiple surgical techniques that have been shown, in combination or as a monotherapy, to successfully treat infection stones [18]. Percutaneous

nephrolithotomy (PCNL) is the mainstay approach and is listed as the first line therapy in both the AUA and EUA guidelines for the treatment of infectious staghorn calculi [8, 19]. Multiple studies have compared extracorporeal shock wave lithotripsy (SWL), ureteroscopy (URS), and anatrophic nephrolithotomy/pyelolithotomy to PCNL and have found inferior stone-free rates (SFR) or greater morbidity associated with the procedure. One of the first comparative trials between PCNL and SWL as monotherapies showed higher SFR of 84.2% with PCNL compared to 51.2% ($p < 0.0001$) for large stones with mean surface area 1378.3 mm^2 (approximately 10 mm radius) [20]. Similar findings were noted in a prospective randomized trial comparing SWL with and without PCNL. SFRs were significantly better with PCNL (77% vs 22% $p = 0.005$) with fewer mean number of anesthetic procedures at 1.96 vs 2.37 ($p = 0.16$). Additionally, the duration of therapy was significantly longer with SWL alone (6 vs 1 month $p = 0.0006$) and the complication rate was significantly higher (15 vs 2 septic complications $p = 0.007$). The most common complications associated with SWL monotherapy for infection stones include urinary sepsis, steinstrasse, acute urinary obstruction, colicky renal pain, and perinephric hematomas. Robust clinical data has resulted in the recommendation that SWL monotherapy should be reserved for stone <20 mm in a non-dilated collecting system or a system decompressed with a ureteral stent or nephrostomy tube [8].

Ureteroscopy, as a stand-alone treatment for infectious stones has no supporting data. One author suggested that patients with partial staghorn calculi and multiple comorbidities could be offered staged flexible URS; however, limited data is available on long-term outcomes of this approach. Despite lack of primary data, URS has emerged as a durable adjunct to primary PCNL and for post PCNL residual stone retrieval. Marguet et al. showed that simultaneous flexible URS and PCNL achieved stone free results in five out of seven patients with an average stone burden of 666 mm^2 (100–2700 mm^2) through a single access in less time (142 vs 166 min $p = 0.36$) compared to their multiaccess experience [21]. Additionally, advances in flexible nephroscopy and URS along with nitinol basket and holmium laser technology have extended the limits of renal calculi treatment through single access PCNL (Fig. 7.2). Wong et al. were able to render 49 patients, with ≥5 cm of stone burden, stone free after an average of 1.6 procedures through a single percutaneous access. Mean operative times for the primary PCNL was 174 and 63 min for the patients requiring a secondary PCNL for an approximate total mean operating room time of 200 min. In this series the patients suffered minimal complications including fever (12.2%), hydrothorax, leg cellulitis, atrial fibrillation, and non- cardiac chest pain (each- 2.8%) with an average hospital stay of 2 days [22]. Lastly, with improvements in the size and clarity of the visual field provided by digital flexible ureteroscopes, URS is succeeding as an adjunctive procedure to remove residual stone fragments after PCNL [23].

The recent interest in laparoscopic anatrophic nephrolithotomy or pyelolithotomy (L-ANPL) stems from prior publications reporting a nearly 100% SFRs [24] with open anatrophic nephrolithotomy (AN), but with the prospect of utilizing the robotic platform to significantly reduce the morbidity of AN [25]. The authors of a meta-analysis comparing L-ANPL to PCNL reported a SFR of 97.57% (362/371) and 87.92% (364/414) accrued across 14 trials including 6 RCTs. Additionally, the study reported significantly lower transfusion rates (OR 0.28, 95% CI 0.13–0.61,

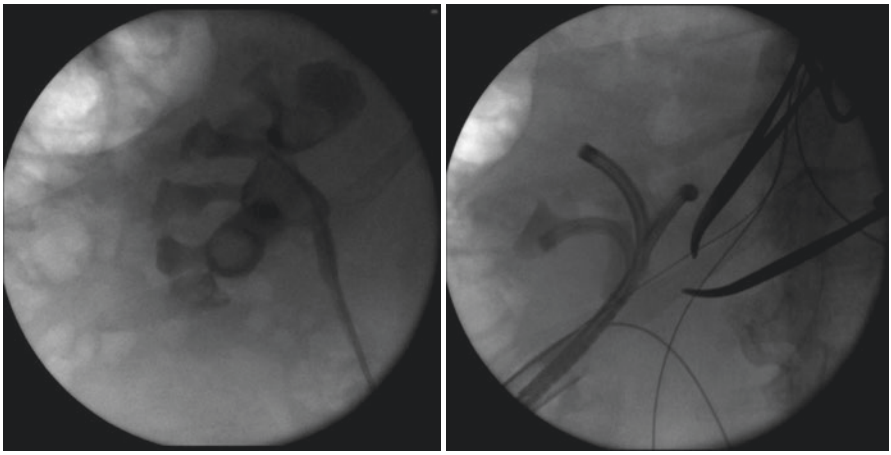

Fig. 7.2 This is an example of a retrograde pyelogram with a complete staghorn. The second image exemplifies the versatility of flexible nephroscopy by layering fluoroscopic images of the flexible nephroscope in each papilla

p = 0.001) and fever (OR 0.38, 95% CI 0.21–0.68, p = 0.001). There was no difference in prolonged urine leakage (OR 1.06, 95% CI 0.49–2.30, p = 0.88) and no reported episodes of sepsis or septic shock [25]. In the same study PCNL was associated with shorter operative times and hospital stays (random-effect model; MD 32.86, 95% CI 12.85–52.86, and MD 0.33, CI 95% −0.24 to 0.89, p = 0.001). These results led the authors to suggest that while PCNL is suitable for many cases L-ANPL is safe and effective and should be considered an alternative management option for appropriately selected cases. Despite these findings, both the EAU and AUA guidelines recommend L-ANPL be limited only to the treatment of nephrolithiasis with a concomitant calyceal diverticulum, an unfavorable body habitus, and a pelvic or transplant kidney [8, 9, 19]. Such recommendations reflect individual study findings which found very low stone free rates using such an approach [26].

Percutaneous nephrolithotomy as a monotherapy remains standard of care for partial and full staghorn infectious stones. Preoperative planning to treat these infection stones with PCNL requires a non-contrasted CT. A CT provides critical information on the stone size, location, and number. Additionally, a CT gives valuable information about anatomic abnormalities and proximity of the kidneys to surrounding organs before surgery [8]. Patients with suspected infections stones should also be evaluated for upper urinary tract obstruction. Decompression and antibiotics prior to PCNL have been shown to reduced rates of SIRS/Sepsis to zero after PCNL. Patients with obstructing and infectious stones displayed SIRS/Sepsis symptoms after PCNL in 6% of cases [27]. If a culture from the renal pelvis is obtained prior to definitive stone surgery, culture specific antibiotic therapy should be started. There is a strong correlation between stone and renal pelvis culture; however, the same does not hold true between stone [28] and bladder urine culture. The discordance between urine and stone cultures is demonstrated by Paonessa et al. in 776 patients of which 75 (9.8%) had a negative urine culture with a positive stone culture and 103 (13.3%) patients had differing pathogens in

their urine and stone culture [29]. This information has prompted the argument for empiric preoperative antibiotics. In one study, patients who were given a 7 day course of nitrofurantoin before PCNL had significantly lower rates of postoperative SIRS (19% vs 49%), positive kidney cultures (0% vs 10%), and positive stone cultures (8% vs 30%) compared to those receiving only pre-induction antibiotics [28]. Antibiotic stewardship remains a priority, which is emphasized by the current AUA recommendations that in the setting of a negative urine culture, only pre-induction antibiotics are necessary [9, 30]. Despite retaining specific fundamentals of percutaneous renal access, PCNL has undergone several modifications in surgical technique with the hope of improving SFR, whilst reducing morbidity of the procedure. Supine PCNL exemplifies modifications, in this case, to reduce cardiopulmonary stressors of prone anesthesia as well as infectious complications like SIRS/sepsis/septic shock by reducing intra-renal pressures. Two meta-analyses evaluating supine versus prone PCNL concluded that SFR ranged from 72.9–83.5% to 77.3–84.5% respectively with an odds ratio of 1.36 (95% CI 1.19–1.56; $p < 0.001$) favoring prone positioning [31, 32]. Currently, to date there are no studies suggesting reduced rates of complications with either approach. Additionally, prior data showing an association between fever after PCNL with sustained intrarenal pressures greater than 20 mmHg [33] has been used to support the argument that supine PCNL has a lower risk of postoperative fever and sepsis; however, to date, there is no evidence to support this proposal.

After PCNL, the standard of care is to obtain a non-contrasted CT of the abdomen to evaluate tube placement relative to surrounding organs, evidence of intra-renal hemorrhage or perinephric hematoma, and stone free status. A consensus on the treatment of residual fragments of infection stones is lacking. Rates of UTIs, readmissions and stone growth for patients with residual infection stone fragments ≥0.4 cm after PCNL were 64% vs 38%, 63% vs 35%, and 88% vs 12% ($p < 0.05$) compared to those without residual fragments [18]. Such results emphasize the importance of secondary procedures in the setting of infection stones to render the patient stone free. It should be noted that complications after PCNL are higher with infection stones compared to non-infectious stones including: fever (10–30%), sepsis (1–5%), and bleeding (8%)/transfusions (6–17%) [34]. Lastly, there is a paucity of literature on antibiotic choice and duration in patients with positive stone cultures with or without residual stones. Antibiotic stewardship is a priority; however, avoiding further stone events is achieved with the sterilization and prevention of bacilluria along with a robust metabolic workup and counseling on the lifestyle techniques to prevent further stone formation.

The key points when managing patient with infection stones include:

- Immediate stabilization of septic patients with antibiotics and decompression of the collecting system when concerned about urinary obstruction.
- When moving forward with an asymptomatic patient with an infection stone, a balanced approach is important; however, there should be an emphasis on getting patients to surgery.
- Rendering the patient stone free is the priority for patients with infection stones to minimize further infections, admissions, stone regrowth and stone events.
- There is no consensus on the length or antibiotic treatment before or after surgical stone removal for infection stones.

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8 The Use of Probiotics and Other Microbiota Therapies to Mitigate Recurrent Calcium Oxalate Stone Formation

Liam Brennan, Kaitlin F. Al, Jennifer Bjazevic, Hassan Razvi, and Jeremy P. Burton

Introduction

The potential role of bacteria in calcium oxalate (CaOx) stone formation has long been debated. While there have been reported correlations between the presence of oxalate-utilising bacteria, and more recently bacterial associated risk factors identified such as antibiotic use, and the development of stone disease; the mechanisms involved in this process are far from elucidated. In the meantime, the incidence of kidney stones continues to increase, and few new options for prevention and treatment have emerged. Given that this disease takes time to develop and the rapidly emerging connections of the microbiome with metabolic diseases, we need to

L. Brennan · J. P. Burton (✉)
Division of Urology, Department of Surgery, Schulich School of Medicine, London, ON, Canada

Department of Microbiology & Immunology, University of Western Ontario, London, ON, Canada

Canadian Centre for Human Microbiome and Probiotics, Lawson Health Research Institute, London, ON, Canada

Lawson Health Research Institute, London, ON, Canada
e-mail: Jeremy.Burton@LawsonResearch.Com

K. F. Al
Department of Microbiology & Immunology, University of Western Ontario, London, ON, Canada

Canadian Centre for Human Microbiome and Probiotics, Lawson Health Research Institute, London, ON, Canada

J. Bjazevic · H. Razvi
Division of Urology, Department of Surgery, Schulich School of Medicine, London, ON, Canada

Lawson Health Research Institute, London, ON, Canada

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consider developing more therapeutic options that potentially support bacteria. This may be in the form of: persevering our indigenous microbiota by reducing antibiotic use, giving conventional probiotics to assist in gut barrier function for more effective handling of oxalate or, administering bacteria which have the ability to metabolise oxalate from diet and potentially endogenous metabolic sources. In this chapter we provide the background and rationale for some of these therapies in the potential future management of kidney stones.

The Role of CaOx in Stone Disease

Kidney stone disease, known as urolithiasis, occurs when a calculus (stone) occurs in the urinary tract. Stones typically form in the kidney and will pass through the body in the urinary system. While smaller stones can pass through without generating any symptoms, larger stones (greater than 5 mm) can block the ureter and potentially result in significant pain, renal damage, or serious infection [1]. The stones are composed of crystals integrated with various proteins, with the crystals forming from a number of different solutes that exist in the urine [2].

There are five major urinary stone compositions including CaOx/calcium phosphate, uric acid, struvite, pure calcium phosphate and cystine. CaOx is the most common primary component and found in approximately 75% of all urinary stones [3]. While the exact cause of CaOx stone formation is not fully elucidated, a number of risk factors are implicated that can drive the formation of CaOx stones. These include genetics, obesity, dietary factors including inadequate fluid intake, various medical conditions including primary hyperparathyroidism and iatrogenic causes. One of the most common risk factors is hyperoxaluria, which is the excessive secretion of oxalate into the urine [4, 5]. Urine oxalate levels are influenced by intake of oxalate-rich foods, intestinal absorption and endogenous production. The interaction between dietary calcium and dietary oxalate is well-known to affect oxalate absorption from the gut. Under normal situations, calcium typically binds to oxalate in the gastrointestinal tract and prevents it from being absorbed into the blood stream. As calcium intake decreases, the levels of free oxalate in the gut are greater, resulting in heightened absorption into the bloodstream and leading subsequently to excretion in higher concentrations by the kidneys into the urine [6]. In addition to this, while in the urine, oxalate acts as a potent inducer of CaOx precipitation. It is now well-established that low dietary calcium or high dietary oxalate are both factors that may result in CaOx stone formation [7, 8].

Treatment of CaOx Stone Disease

There are a number of therapies that exist to treat CaOx stones. Shockwave Lithotripsy (SWL) is one of the more common treatments for stones, involving the use of high-energy shockwaves to break the stones and allow passage of the fragments [9, 10]. While it is a non-invasive procedure and easily administered, there

are several caveats to its usage. Not all stones can be fragmented, and factors such as stone size and location, and certain patient characteristics might favour other treatment modalities [11]. The most commonly utilized treatment for urinary stones is flexible ureteroscopy and Holmium: YAG laser lithotripsy. This minimally invasive therapy allows effective fragmentation of stones regardless of composition. Another potential treatment of urinary stones is percutaneous nephrolithotomy, and is generally reserved for patients with large stone burdens or for those with complex anatomical issues that preclude the use of SWL or ureteroscopy [12].

While these procedures can result in the successful treatment of the stone, recurrence rates are high, with 60% of patients suffering from recurrence within 5 to 10 years of their initial stone event [13, 14]. This can be largely attributed to the fact that removing the stone does not treat the cause of the stone formation. In the case of CaOx stones, if a patient had hyperoxaluria that was not corrected, the persistent hyperoxaluria would continue to drive future stone formation [15, 16]. As mentioned previously, abundance of dietary oxalate may contribute to CaOx stone formation. While some patients are advised to alter their diets to avoid oxalate rich foods in an attempt to prevent stone recurrence, the long-term effectiveness of this is poor, and any additional side effects from altering their diet remains uncertain. However this does not explain why certain populations, such as vegetarians, who consume relatively high oxalate levels do not suffer a greater burden of kidney stones [3]. The way we respond to oxalate in our diet is unclear, and patients with kidney stones may respond differently based on the health and integrity of their intestinal barrier. Therefore, other methods that would either prevent intestinal absorption of oxalate or reduce oxalate levels in the gut would be ideal for preventing recurrent stone formation.

Microbiome Damage and Stone Formation

Given the evidence in the literature that supports the beneficial presence of organisms such as *Oxalobacter formigenes* (*O. formigenes*) in reducing urinary oxalate excretion and CaOx stone formation; it is also important to discuss the impact that disrupting the patient's microbiome may have on the development of stone disease. If the microbiome is in fact involved in kidney stone disease, one would expect there to be a relationship between the disease and substances which disrupt or alter microbial populations, such as antibiotics. Disruptions to the microbiome have been well studied in metabolic syndromes such as obesity and diabetes. There appears to be some correlation in the United States between where antibiotics are more frequently administered and the prevalence of these diseases, which implies that the microbiome may be implicated [17–19]. There also appears to be some overlap between high rates of antibiotic prescriptions and nephrolithiasis in the south-eastern United States [20–22]. However, it has also been suggested that these "belts" of stone disease are related to dehydration; as urinary solute precipitation is accelerated with decreased urine volume. Similarly, it has been postulated that the rise in kidney stone disease may be associated with global warming [20, 23]. It is difficult to

ascertain whether these are just generally ailing populations, or whether their microbiomes have in fact been disrupted by antibiotic use, leading to stone formation and metabolic dysfunction.

In a more direct fashion, a recent study by Tasian et al. has shown significantly increased odds of urinary stone disease with the use of five classes of antibiotics [24]. In an impressive study looking at over 285,000 healthy controls and nephrolithiasis patients, they determined the association between 12 oral antibiotic classes and stone disease. Specifically, significantly increased odds of stone disease were associated with sulfas, cephalosporins, fluoroquinolones, nitrofurantoin/methenamine, and broad-spectrum penicillins. These effects were most significant with antibiotic exposure occurring more recently, and at younger ages.

The incidence of nephrolithiasis for children also appears to be increasing rapidly, specifically in calcium-based calculi [25]. The reason why this is occurring in children is unclear; some have suggested this is related to increasing body mass index (BMI), salt consumption, or decreased calcium and water intake [26]. A mouse study modeling pediatric antibiotic treatment with either beta-lactam or macrolide antibiotics demonstrated altered host and microbiota development; with a decrease in the relative abundance of oxalate metabolism genes, and faecal oxalate levels [27]. Recent studies also suggest nephrolithiasis is up to four times more common in children with asthma, a condition that has links with early childhood antibiotic use [28]. In addition, asthma is also commonly treated with steroids, and glucocorticoid-mediated alterations on gut microbiota are known to occur. The microbiome studies of the future will have to better control for antibiotic substances (most recent usage, antibiotic class, use of sanitizers and detergents), as some antibiotics can have extremely long-term effects on the microbiome [29, 30].

In adults, a study looking at the occurrence of *O. formigenes* showed that patients treated with antibiotics for *Helicobacter pylori* had decreased detection of this important oxalate-utilizing bacterium [31, 32]. Of four *O. formigenes* strains tested to commonly used antibiotics, all were resistant to amoxicillin, amoxicillin/clavulanic acid, ceftriaxone, and vancomycin and at least one strain to nitrofurantoin [33]. While these organisms may be moderately resistant to commonly used and often penicillin-based antibiotics, it is harder to say the effect of selectively depleting other supportive or symbiotic bacterial types.

It is well known that microbiome alterations in the colon can affect the intestinal environment at various levels, leading to changes in gut permeability [34]. This can increase the absorption of oxalate and allow inflammatory bacterial components to enter the body [35]. Hatch et al. identified that in rats with chronic renal failure there was an adaptive response in the colon, which "dumped" more oxalate due to the failure of the kidneys [36]. This may be a future therapeutic strategy for the hyperoxaluric stone patient, however the question remains if we can up-regulate this dumping response in humans, and if so, how the microbiome would be involved.

Many mechanisms could be leading to these observed relationships with antibiotics and stone disease. It is possible that the antibiotics deplete the ability of the gut microbiome to degrade and maintain oxalate homeostasis. Alternatively, direct crystallization of various antibiotics can occur, and this precipitation occurring in

the kidney may act as a stone nidus [37]. Finally, the over- and mis-use of antibiotics could be leading to antibiotic resistant uropathogens in the bladder, which may ascend to the kidney, inciting inflammation and stone disease. Future studies should aim to elucidate the underlying causes, and meticulously detail antibiotic use in microbiome studies of stone patients going forward.

Environmental Damage to the Microbiota

Beyond the over subscription of antibiotics, Western society is in general, heavily medicated and bombarded with pharmaceuticals on a daily basis. In the United States, an average of over 11 prescriptions were filled per person per year in 2011 [38]. Exposure to pharmaceuticals can result from consumption of one's own prescription or over-the-counter medication, but also inadvertently through the drinking water [38, 39].

It is now known that drugs originally developed to target human cells rather than microbes can alter the microbiome, and while many such drugs often have gastrointestinal side effects, the direct effect of such drugs on the microbiome is rarely investigated. In exception to this, Maier et al. completed an extensive screening of more than 1000 non-antibiotic marketed drugs against 40 representative gut bacterial strains. They found that almost a quarter of the tested drugs inhibited growth of at least one of the tested strains [40].

In addition to altering the microbiome, several of these environmentally acquired drugs can also lead to nephrolithiasis directly. For example, furosemide is a loop diuretic that can be detected in Canadian drinking water and is known to promote CaOx stone formation [39, 41]. Outbreaks of food-based melamine contamination have also been known to cause melamine-uric acid stones [42, 43]. Pharmaceutically active compounds detected in the environment should be a priority for future study with regards to microbiome interactions, and stone disease.

Microbiome Restoration Therapies

While the evidence of bacterial involvement in kidney stones is still emerging, it appears as though future preventative therapies will need to account for bacteria playing a role in the pathology of the disease. For instance, if childhood antibiotic use is determined to be a contributor to the condition, an objective of future treatments would be to cultivate or reacquire a beneficial microbiome. We obtain most of our bacteria externally, starting from our mothers, and family members and then through our food and environment. In fact, while many factors determine the composition of the gut microbiome, diet has been shown to change its composition in as little as 2 days [44]. If high oxalate levels increase the risk of kidney stones, how does the microbiome adapt to an increase in dietary oxalate? Studies suggest that the microbiota is an important factor allowing humans to quickly adapt to altered diets, facilitating dietary diversity [44, 45].

Probiotics

Probiotics are live, non-pathogenic microorganisms that when administered are able to confer some type of health benefit to the host [46]. Probiotics can either be taken in forms such as capsules that are similar to other drug delivery systems, or can be taken by consuming various probiotic foods, such as yogurt. Other fermented foods which are not strictly considered probiotics, such as sauerkraut or kombucha, may also offer some benefits by lowering oxalate levels through bacterial metabolism; however, they currently lack evidence-based studies. This original concept is typically attributed to Élie Metchnikoff, who theorised that Bulgarian peasants had longer life expectancies due to the fact that the consumption of yogurt was a staple of their diet [47].

While probiotics are typically thought to either reinforce or restore the normal microbiota in an effort to promote ongoing quality of health, there are increased instances of clinicians using probiotics to treat specific medical conditions, including diarrhoea [48]. Their exact mechanism of action has yet to be fully determined, as in reality most probiotics strains pass through the intestinal tract without colonising. However, they are thought to improve gut barrier function and reduce intestinal "leakiness" associated with chronic conditions. Given the broad spectrum of applications that probiotics appear to have, it is only reasonable to assume that their usage could also be applied to clinically treat CaOx stone formation, perhaps through reducing intestinal paracellular oxalate absorption, increasing metabolism or enzymatic degradation of oxalate, or by promoting other members of the microbial community which perform this function.

O. formigenes; the Bacterial Proto-oxalate Utilizer in Humans

O. formigenes is a gram-negative, obligatory anaerobic, rod or curved shaped bacterium that colonises the gastrointestinal tract of vertebrates, including humans [49, 50]. *O. formigenes* is not the only bacteria that can metabolise oxalate, but in fact utilizes it as its primary carbon source, using the oxalyl-CoA decarboxylase enzyme [51, 52]. The implication of this is that in humans that are colonised with *O. formigenes*, there is less free oxalate available to be absorbed in the gut as the bacteria utilise it, therefore less is excreted in urine, reducing the risk of CaOx stone formation.

A number of case-control studies have suggested that subjects that were colonised with *O. formigenes* had a reduced risk of developing CaOx stone disease [53–55]. These studies demonstrated that cohorts of stone formers have less intestinal *Oxalobacter* when compared to the control groups. They also indicated that urinary oxalate excretion was lower in patients that were colonised when compared to un-colonised patients. However, these studies did not control for differences in dietary oxalate. Another study that did control for dietary oxalate also demonstrated not only a reduction in urinary oxalate in patients colonised with *O. formigenes* when compared to those who were un-colonised, but also a reduction in plasma

oxalate as well [56]. There was also an observation that colonisation with *O. formigenes* was associated with a significant reduction in stone episodes. These studies would suggest there is potential to use *O. formigenes* as a probiotic treatment for CaOx stones.

While there has been a relatively extensive number of studies looking at the presence of *O. formigenes* in stone patients, there have been very few that have investigated the probiotic application of *O. formigenes*. One in vivo study in a rat model with induced hyperoxaluria demonstrated that administering *O. formigenes* orally resulted in a significant reduction in urinary oxalate [57]. Rats that received higher doses of *O. formigenes* had almost no detectable oxalate excreted in the urine. Another study performed in rats demonstrated that similar to human studies, rats that are naturally colonised with *O. formigenes* had reduced urinary oxalate levels, and that the *Oxalobacter* interacted with the colonic mucosa at a physiological level to promote oxalate excretion [58]. The same group also demonstrated that in a mouse model deficient in liver alanine-glyoxylate aminotransferase, which exhibits hyperoxaluria, colonization with *O. formigenes* reduced urinary oxalate levels [59]. This reduction in urinary oxalate could also be seen in the group without disease as well, demonstrating it is not an effect that is only observed in the disease state. Another murine study demonstrated that in both germ free mice and mice with a defined altered microbiota, *O. formigenes* could stably colonise the gut in the absence of other organisms and was still able to reduce urinary oxalate levels [60]. These data suggest that *O. formigenes* can potentially colonise the gut with limited impact on the existing composition of the microbiome.

Clinical studies of the effects of *O. formigenes* in stone patients are even less extensive than the animal studies. Administration of *O. formigenes* HC1 to humans demonstrated a prolonged colonisation in the gut and a reduction in urinary and stool oxalate levels following administration of an oxalate load [61]. Another study also showed that urinary oxalate can be reduced following supplementation with *O. formigenes* in patients with both normal renal function and in renal failure [62]. One randomised study in humans demonstrated that administering *O. formigenes* twice a day reduced hyperoxaluria at 1 month following the beginning of the treatment, while the control group demonstrated no change in urinary oxalate levels [63]. Both groups demonstrated similar levels of other urinary abnormalities, such as hypercalciuria; which suggests that the mechanism of *O. formigenes* is specific to reducing the levels of urinary oxalate.

The use of *O. formigenes as a clinical probiotic* is a promising potential candidate treatment for stone disease. All of the evidence suggests that being naturally colonised with *O. formigenes* leads to less urinary oxalate, and studies looking at colonising animals and humans with it demonstrate the same result. As hyperoxaluria is a significant risk factor for the formation of CaOx stones, the ability to safely reduce the levels of oxalate in the urine of recurrent stone formers is an enticing target for treating patients. Further understanding of *O. formigenes* growth requirements to maintain long-term colonisation in vivo, and proving that administering it is safe to all humans are required to drive the development of this as a potential treatment for recurrent CaOx stone formers.

Oxalobacter Replacement

As detailed above, there are many studies that support the potential role that *O. formigenes* plays in degrading oxalate and enhancing its intestinal secretion; epidemiological evidence also supports its existence in 'healthy' people [61]. Studies have investigated the use of *Oxalobacter* as a probiotic, but surprisingly there haven't been many studies in large cohorts that include *O. formigenes* supplementation while simultaneously controlling dietary oxalate intake [61, 63]. Although some stability and delivery studies have been performed on *O. formigenes*, its relatively limited use in human trials may be attributable to regulatory concerns when delivering a live microorganism without a history of use in foods to humans [64]. While the organism is considered a strict anaerobe, it does appear to exhibit tolerability to bile salts and low pH, indicating *O. formigenes* could survive through the harsher regions of the gastrointestinal tract [61]. Although it is aimed at treating hyperoxaluria, the *Oxalobacter*-containing probiotic Oxabact® may be one such product Urologists lean on in the future of kidney stone treatment and prevention.

Lactic Acid Bacteria-Based Probiotics

Lactic acid bacteria are an order of gram-positive, facultative anaerobic or microaerophilic, rod or cocci shaped bacteria. These bacteria typically produce lactic acid as a major metabolic end product of carbohydrate fermentation and are usually involved in various steps of food fermentation. They also constitute a large number of the microbiota at various tissue sites in humans, and for these reasons are generally recognized as safe for administration.

Some species of lactic acid bacteria are able to degrade oxalate, though to a much lesser degree than *O. formigenes*. One study administered a combination of lactic acid probiotic bacteria to patients with hyperoxaluria and demonstrated a reduction in urinary oxalate levels both 24 h and 1 month after treatment [65]. While the strains of bacteria used in the treatment differed in their ability to survive and degrade oxalate in vitro, there was no evidence of them expressing known oxalate degrading genes. Another study demonstrated that in patients that were considered high oxalate absorbers, the probiotic VSL #3, which contains many lactic acid bacteria, was able to reduce the amount of oxalate excreted in the urine [66]. Contrariwise, another study demonstrated no difference in urinary oxalate levels when treating patients with lactic acid bacteria probiotics [67]. The fact that these studies have shown both positive and negative outcomes when testing non-*Oxalobacter* probiotic strains as therapy for patients with kidney stones is unsurprising. It is important that probiotic studies be evaluated on the basis of strain, dose, and format. It is not yet possible to determine whether this potential treatment has value due to the small numbers of patients, lack of follow-up, and broad preparations that have been used in the studies thus far. The lack of a clearly defined mechanism of oxalate degradation, like that found in *O. formigenes* also means that the mechanism that these bacteria might use to reduce oxalate is unknown. Further

studies need to be done to identify the optimal combination of lactic acid bacteria to be used in a potential probiotic formulation. Although yet inconclusive, these studies have merit and deserve much further investigation. Not only could probiotics degrade dietary oxalate before absorption, but they could also stabilize intestinal barrier integrity, a factor known to be critical in the luminal secretion of oxalate during host-mediated detoxification [50, 68].

Engineered Microbial Solutions

The engineering of organisms to attain desired attributes to reduce oxalate in the gastrointestinal tract by either producing enzymes directly or through potential microbial delivery is possible. Oxazyme® is a non-systemic orally delivered drug composed of recombinant oxalate decarboxylase for the treatment of primary hyperoxaluria. In vitro studies have shown that Oxazyme® can degrade oxalate in both simulated gastric and small intestinal environments, acting as an intercept therapy for the management of dietary oxalate prior to absorption [70]. *Bacillus subtilis* contains the oxalate decarboxylase gene *Yvrk*, and has also been investigated for its functionality in oxalate nephrolithiasis treatment. An *Escherichia coli* strain expressing the *Yvrk* from *B. subtilis* was developed and successfully degraded oxalate in vitro; furthermore, purified enzyme from the recombinant *E. coli* showed oxalate degradation ability in a rat model of hyperoxaluria [71, 72].

There has also been work undertaken looking into novel, recombinant probiotic organisms for the prevention of CaOx stone formation. One group engineered a recombinant *Lactobacillus plantarum* that expressed the oxalate decarboxylase enzyme, allowing it to degrade oxalate and reducing urinary oxalate and crystal formation in rats in vitro [69]. While these studies do further support the idea that the degradation of excess dietary oxalate is important for preventing CaOx stone formation, these recombinant organisms are unable to be used as current food and drug safety administration laws stipulate that probiotics cannot be genetically modified.

While these studies demonstrate it is possible to design and engineer strains to produce oxalate-degrading enzymes, it is undetermined if they will promote the intestinal secretion of oxalate. There are likely other factors that impact how *O. formigenes* has adapted to handle oxalate in the colonized human gut, and it is unclear if recombinant bacteria, probiotics or purified enzymes would provide long-term therapeutic value to oxalate nephrolithiasis patients.

Fecal Microbiome Transplantation (FMT)

From studies in animals, we know that improved oxalate degradation may be achieved after whole community microbial transplants. Miller et al. showed that an FMT from the wild mammalian herbivore *Neotoma albigula* into laboratory rats resulted in significant increases in oxalate degradation, an effect that persisted up to

9 months after the initial transplant [73]. The selection of *Neotoma albigula* was important as it is uniquely attuned to consume a diet high in oxalate (up to 9% dietary oxalate by weight), a phenotype that, as in all mammals, is conferred exclusively by the gut microbiota as opposed to mammalian enzymes. This approach may be utilized as a future treatment for CaOx kidney stone disease in humans.

Currently, the primary use of FMT in humans is for recurrent *Clostridium difficile* infection, with reported success rates of up to 90%. Currently in Canada, FMT is performed on a routine basis for *C. difficile* infections and is proving to be one of the best treatment options [74]. FMT is now undergoing study in humans and animals for its potential use in extra-intestinal diseases, including metabolic syndrome, non-alcoholic fatty liver disease, and even multiple sclerosis [75, 76]. Of interest, in a study investigating metabolic syndrome in a small number of participants, those that received an FMT from a lean donor often restored keystone microbes, including *O. formigenes* [77]. This treatment method may be promising for nephrolithiasis because of the known role of intestinal bacteria in oxalate degradation, barrier function, and oxalate secretion. While introduction of *O. formigenes* or other single-strain probiotics to a "dysbiotic" microbiome may only have short-term effects, an FMT could show promise as a more potent form of microbiome modification and treatment.

A potentially more regulated approach to the FMT would be strategic microbiome reconditioning [78]. A "dysbiotic" microbiome from a diseased individual may be collected, reconditioned for specific functions *ex vivo*, then reintroduced to the patient [74]. This could be achieved by culturing the original sample in fermenters, or chemostat systems, that have been pulsed with specific substances in order to increase the relative abundance of bacterial groups of interest. Specifically, culturing of a patient sample in the presence of oxalate may offer a way to restore oxalate-degrading species to higher abundance. The benefit of this system would be that patients are receiving modified autologous transplants, thereby minimizing the risk of receiving any unwanted phenotypes that sometimes occur in allogeneic transplants [79].

Conclusion

While there is a large body of work to support the use of probiotics and microbiome reconditioning tools to limit recurrent stone formation in patients, the field is still very much in its infancy. Further studies need to be undertaken in patient cohorts in order to establish if these treatments are efficacious in large populations and prevent stone formation over a long duration.

Despite this, these potential therapies are a promising avenue to pursue. Further investigation into these will improve the understanding of both the involvement and importance of the host microbiome in stone disease, which in turn will further enhance the types of microbiome-based therapies that are available. The relative safety of administering these therapies is attractive in order to limit the active disease burden in stone patients and the cost of the therapy is relatively inexpensive when compared to traditional drug therapy. Used in

combination with traditional urology practices, probiotic treatment and other microbiome therapies could potentially ablate and prevent recurrent stone formation in patients that suffer from CaOx stones.

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9 Role of *Oxalobacter formigenes* Colonization in Calcium Oxalate Kidney Stone Disease

John Knight and Ross P. Holmes

Microbiology of *O. formigenes*

*O. formig*enes is a Gram-negative, obligately anaerobic, rod or curve-shaped, non-motile, non-spore forming bacterium that belongs to the *Betaproteobacteria* class and *Burkholderiales* order. Its existence was first recognized from its role in acclimating livestock to the ingestion of high-oxalate diets and preventing oxalate toxicity [1, 2]. Comparisons of the profiles of cellular fatty acids of 17 strains of *O. formigenes*, including strains isolated from gastrointestinal contents from humans, sheep, cattle, pigs, guinea pigs, rats and from fresh water lake sediments, support the concept of separating these strains into two main groups (currently designated as Group I and II). In Group 1 strains, a cyclic 17 carbon fatty acid predominates whereas in Group 2 a cyclic 19 carbon acid is dominant [3].

The products from oxalate metabolism are carbon-dioxide and formate, with approximately 1 mole of each produced per mole of oxalate metabolized. Energy generation is centered on the development of a proton motive force through the electrogenic exchange of oxalate (in) and formate (out) across the cell membrane together with the consumption of a proton inside the cell when the CoA-ester of oxalate is decarboxylated by oxalyl-CoA-decarboxylase [4, 5].

The availability of the genome [6] and, more recently, proteome of *O. formigenes* [7] has provided an opportunity to increase our understanding of the biology of this organism and how it survives in its environment. The release of the genome sequence of a Group 1 (OXCC13) and a Group 2 strain (HOxBLS) by the Broad Institute has provided a genetic framework for investigating important biological properties of the organism [6]. An independent sequence for OXCC13 has been

J. Knight · R. P. Holmes (✉)
Department of Urology, University of Alabama at Birmingham, Birmingham, AL, USA
e-mail: knight74@uab.edu; rholmes@uab.edu

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published as well as the sequence of the HC-1 strain which is being used by Oxthera in clinical trials [8, 9]. With this data, molecular and functional studies can now be performed to identify important proteins and pathways that promote colonization resilience, enhance aerotolerance and increase enteric secretion of host derived oxalate. A recent review of the genomic sequences of the two strains of *O. formigenes* identified some interesting differences that may suggest the two strains utilize different pathways to survive and flourish within the intestine [6]. Mass spectrometry based shotgun proteomics identified 1822 proteins of the 1867 unique protein coding genes in the Group 1 *O. formigenes* strain OxCC13 [7]. From the protein datasets presented it is clear this organism contains a repertoire of metabolic pathways that mediate adaptation with nutrient shifts and environmental stress. For example, the proteomic analysis showed superoxide dismutase increases in stationary relative to log phase suggesting *O. formigenes* has the ability to persist outside the anaerobic environment of the intestine.

Growth of *O. formigenes* in culture occurs under anaerobic conditions, with optimal growth at pH between 6 and 7 in a carbonate–bicarbonate buffered medium that contains minerals, oxalate, acetate, and a small amount of yeast extract. It requires a low concentration of acetate (0.5 mM) to grow, but acetate alone cannot support growth [3]. Oxalate serves as both the energy yielding substrate and the major source of carbon for growth [10, 11]. Smaller amounts of carbon are also assimilated from acetate and carbon dioxide. The energy yield from oxalate is low, but sufficient to support growth. The low yield of *O. formigenes* in culture and its sensitivity to oxygen has implications for the preparation of *O. formigenes* for probiotic use. A recent study examining some common processes and conditions associated with manufacturing of probiotic strains highlighted the resilience of the Group 1 *O. formigenes* strain OxCC13 to lyophilization and storage in yogurt. In light of this work, it would be of interest to test if individuals can be colonized with either lyophilized *O. formigenes* or *O. formigenes* mixed in with yogurt. Previous *in vitro* work examining various biological properties of *O. formigenes* showed that different strains of *O. formigenes* have different tolerances to environmental stress such as acid and air exposure. This work would suggest that it will be prudent to identify an *O. formigenes* strain more resilient to common processes associated with probiotic preparation before embarking on a colonization study in humans.

A recent in vitro study indicated that *O. formigenes* culture conditioned medium stimulates oxalate secretion in human intestinal Caco-2-BBE cells [12]; however, these findings were not replicated by a different group [13] and warrants further investigation. Arvans et al. also showed that rectal administration of *O. formigenes* culture conditioned medium resulted in a reduction in urinary oxalate excretion in a mouse model of PH1; however, a direct measurement of enteric oxalate secretion utilizing oxalate isotopes administered intravenously and then measured in the gut is still needed to quantify the extent of this pathway. Of interest was the recent filing of a patent by OxThera Pharmaceuticals that covers the invention of the isolation and administration of secretagogues derived from *O. formigenes* that may enhance oxalate secretion into the intestinal lumen (http://www.google.com/patents/WO2015002588A1?cl=en). Although the work described in this patent did not identify any compelling secretagogue candidates, the identification of a bioactive

factor or factors secreted by *O. formigenes* that induces oxalate secretion may be an effective therapy to reduce the oxalate burden in patients with Primary Hyperoxaluria.

O. formigenes in the Human Gut

Because of *O. formigenes* dependency on oxalate for growth, its intestinal numbers are sensitive to both dietary oxalate and dietary calcium intake. This was highlighted in a study where *O. formigenes* numbers were measured in the stool of healthy subjects equilibrated to diets controlled in oxalate, calcium and other nutrients, as shown in Fig. 9.1 [14]. In this study, bacterial numbers were shown to increase 12-fold on average as dietary oxalate increased 15-fold. Interestingly, the availability of oxalate was also shown to influence bacterial numbers as a fivefold increase in dietary calcium, which will limit the bioavailability of oxalate due to the high affinity of calcium for oxalate, decreased bacterial numbers approximately fivefold. The dependency for oxalate and the inverse relationship between dietary calcium and *O. formigenes* numbers may lead to a loss of colonization in stone formers who are recommended to maintain an adequate calcium and low oxalate intake and warrants further investigation.

Enumeration in human stool from healthy non-kidney stone forming individuals suggests *O. formigenes* represents a tiny fraction of the total intestinal microbiota [6]. Many low abundance bacteria are thought to survive in the intestines by occupying specific nutrient niches where competition for their food source is limited [15].

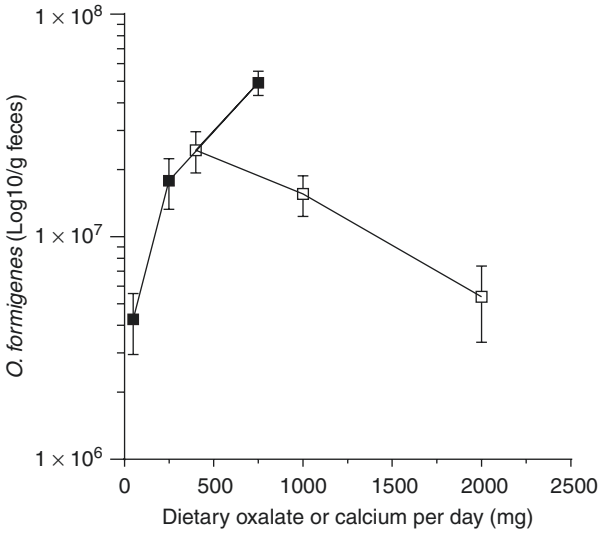

Fig. 9.1 Number of fecal *O. formigenes* with changes in dietary oxalate (■) or dietary calcium (□). Daily calcium intake was 1000 mg on the varied oxalate dietary phase and daily oxalate was 250 mg on the varied calcium dietary phase. Real-time PCR was used to quantitate *O. formigenes* numbers. 5.5×10^4 CFU/ng DNA was used to convert qPCR data to number of *O. formigenes* per g feces. (Modified from [14])

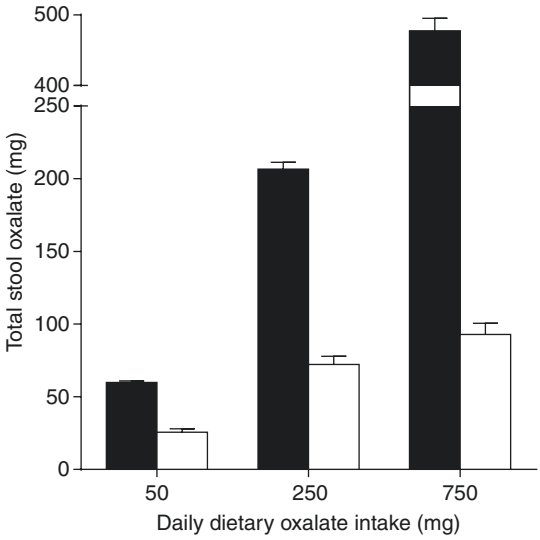

Fig. 9.2 Stool oxalate of *O. formigenes* colonized (□) and non-colonized healthy subjects (■) on nutrient controlled diets varying in oxalate. Daily calcium intake was 1000 mg. (Modified from [14])

Indeed, both *in vitro* culture studies [16] and a recent human study [14] show that *O. formigenes* utilizes oxalate more efficiently than many other bacteria. Thus, an important factor in the survival of this organism in the intestines is its unique ability to outcompete other bacteria for its food source [14], highlighting the highly efficient oxalate degrading capacity of *O. formigenes* relative to other microbiota. These data also show that the oxalate degrading capacity of the microbiome of non-colonized individuals is negligible at low oxalate intake, but increases with adaptation to ingestion of higher levels of dietary oxalate, as the dietary oxalate recovered in stool with a daily intake of 250 and 750 mg dietary oxalate was ~80% and ~60%, respectively (Fig. 9.2). The impact of these "generalist" oxalate degrading bacteria in calcium oxalate stone disease is not known, and warrants careful investigation [17].

O. formigenes Colonization

Little is known about how and when individuals become colonized or how *O. formigenes* persists over time. The source of *O. formigenes* that colonizes the gut is not known. Studies to date suggest it occurs early in childhood [18] and based on what we know about *O. formigenes* transmission from animal experiments it is obtained from the environment, not directly from the mother [19].

A review of the colonization frequencies conducted worldwide indicated that 38–77% of a normal population is colonized and it was consistently observed that the colonization frequency in stone formers was about half that in normal subjects [20–23]. Several studies have indicated that the intake of antibiotics can result in the loss of colonization [21, 24, 25], and this is supported by lower prevalence of *O. formigenes* in both cystic fibrosis patients [26], and calcium oxalate stone formers who are frequently prescribed antibiotics [27, 28]. It is also possible that a lower rate of colonization in stone formers is due to patients restricting dietary oxalate

intake. To date, there has only been one study to examine factors that impact colonization, and in this study [21] only a slight (non-significant) trend was observed between prevalence of colonization (simply whether or not a person was colonized with *O. formigenes*) in normal subjects and dietary oxalate intake.

Recent analyses of the American Gut Project (AGP) large-scale datasets (>8000 samples) has provided novel insights into *O. formigenes* colonization of the human gastrointestinal tract [22]. These analyses support the finding that individuals residing in countries with strong healthcare programs and/or higher economic life-styles tend to have significantly less *O. formigenes* colonization, an observation consistent with the higher general use of antibiotics within these more affluent societies and populations. The AGP sequence analysis also revealed humans may be co-colonized by Group 1 and Group 2 *O. formigenes* strains, and Group 1 strains may be the most prevalent and abundant strains in the human gastrointestinal tract, while group 2 strains are less common; however, studies still need to be performed to determine if colonization with both strains is possible and offers any advantages regarding oxalate handling. The AGP and a recent study of gut microbiota in stone formers and controls [29] emphasize that biodiversity in the communal structure supporting *O. formigenes* is a key feature in oxalate degradation.

The ability to re-colonize individuals lacking *O. formigenes* has previously been addressed by a study in which two healthy adults not colonized with *O. formigenes* became colonized following the ingestion of cultured *O. formigenes* [30], and subsequently remained colonized for 9 months. However, other studies where *O. formigenes* was provided in the form of an enteric coated capsule or as a frozen paste to patients suffering from Primary Hyperoxaluria, resulted in only a minority of the patients remaining colonized post-treatment [31, 32]. Therefore, although it seems quite possible that *O. formigenes* colonization of non-colonized stone formers may be a cheap and effective way to help minimize stone risk in calcium oxalate stone formers, long term colonization studies are required.

O. formigenes Colonization and Risk of Calcium Oxalate Stone Disease

Since the discovery of *O. formigenes* in 1985 and the recognition that it resides in the human gut and degrades oxalate, a role for the organism in stone disease has been considered. Initial case–control studies with small numbers of subjects suggested colonization may be protective against stone disease [27, 33, 34], as measurements of urinary oxalate excretion was lower in colonized compared to non-colonized individuals despite a large variability in oxalate excretion and a lack of dietary oxalate and calcium control during urine collections. In addition, a recent study showed 24 h urinary oxalate excretion and plasma oxalate were significantly lower in *O. formigenes* colonized patients compared to *O. formigenes* negative patients on a standardized diet [35]. Colonization was also found to be significantly inversely associated with the number of stone episodes.

Similarly, the association of recurrent calcium oxalate stone disease and a lack of *O. formigenes* was assessed in a study of 247 calcium oxalate stone formers and 259

matched controls [20]. The odds ratio for forming a recurrent stone when colonized was found to be 0.3, which indicates a 70% reduction in stone risk. Surprisingly, there was no difference in urinary oxalate excretion between colonized and non-colonized individuals in either group, which may be due to highly variable oxalate excretion results despite a large enough sample size, as well as the fact that dietary oxalate and calcium levels were not controlled. The discordance in results may be partially explained by our study in healthy subjects that illustrated that the beneficial oxalate degrading activity of *O. formigenes* is highly dependent on diet [14]. In this study, urinary oxalate excretion was only found to be significantly lower in colonized subjects compared to non-colonized individuals when subjects were administered a low calcium (400 mg/day) and moderate oxalate (250 mg/day) diet, indicating that the efficiency of this bacterium is not maximal at all calcium and/or oxalate concentrations. Further controlled dietary studies are needed to examine what levels of dietary oxalate and calcium intake are required for successful colonization of non-colonized calcium oxalate stone formers with *O. formigenes*.

Patients subjected to Roux-en-Y gastric bypass (RYGB) are at risk of hyperoxaluria and calcium oxalate kidney stone disease [36], most probably due to increased net gastrointestinal absorption. Of note, rates of *O. formigenes* colonization have been shown to be lower in morbibly obese individuals being evaluated for or just after bariatric surgery [37, 38]. Recent work by Canales and Hatch using a RYGB rat model showed that colonization with *O. formigenes* lowered 24 h urinary oxalate excretion 74% in RYGB animals, suggesting patients who exhibit hyperoxaluria after malabsorptive bariatric surgery may benefit from colonization with *O. formigenes* [39]. *O. formigenes* has been demonstrated to induce gastrointestinal oxalate secretion in animal models, which may be a second mechanism by which this organism decreases oxalate levels within the circulation and the kidney [39–42]. A recent controlled dietary study with 11 *O. formigenes* calcium oxalate stone formers and 26 non-colonized calcium oxalate stone formers, showed absorption of a $^{13}C_2$-oxalate load was not significantly different between the groups, but plasma oxalate concentrations were significantly higher in non-colonized (5.79 μmol/l) compared to *O. formigenes* colonized stone formers (1.70 μmol/l) [35]. These data support the findings in rodent models that *O. formigenes* induces enteric secretion of endogenously produced oxalate, thereby decreasing plasma oxalate concentration. Whether the modification of host oxalate transport properties by *O. formigenes* colonization underlies the reduction of risk for calcium oxalate stone formation is currently being tested by OxThera, Inc., in a Phase 3 clinical trial with Primary Hyperoxaluria patients.

Conclusions

Much still remains to be learned about how *O. formigenes* establishes and maintains gut colonization. Unraveling these mechanisms is especially important with respect to the colonization of non-colonized stone formers. Further studies on the factors involved in colonization resilience and enteric secretion of host derived oxalate are warranted in light of this. The range of conditions where *O. formigenes* lowers stone risk and the role the composition of the gut microbiome plays in this remain to be clearly defined.

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BCG for the Treatment of Non-muscle Invasive Bladder Cancer

10

Roland Seiler and Peter C. Black

Background

The History of BCG

BCG was developed as a vaccination against tuberculosis, the most common cause of death in the nineteenth century. *Mycobacterium bovis* was isolated from the milk of an infected heifer and transferred to Albert Calmette and Camille Guérin in the Pasteur Institute in Lille. After 239 passages in culture between 1908 and 1921, the initial extremely virulent strain became avirulent. In 1921 the first BCG vaccination was successfully given to a newborn girl. After dissemination of this vaccination, mortality from tuberculosis decreased dramatically from 25–43% to 1.8%.

Early Trials of BCG in Cancer Therapy

Pearl noticed in 1921 a reduced incidence of cancer in the autopsies of patients who suffered from tuberculosis [1]. Holmgren subsequently became the first to treat stomach cancer with BCG and reported successes in 1935 [2]. The anti-cancer effect was thought to be due to the profound stimulation of the reticuloendothelial system (also known as mononuclear phagocyte system) by BCG. It was not until

R. Seiler
Department of Urology, University Hospital Bern, Bern, Switzerland
e-mail: roland.seiler@insel.ch

P. C. Black (✉)
Department of Urologic Sciences, University of British Columbia, Vancouver Prostate Centre, Vancouver, BC, Canada
e-mail: pblack@mail.ubc.ca

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after this application in patients that the first landmark studies in animal models were reported in 1959 [3]. Further uncontrolled clinical testing revealed that BCG could lower the incidence of leukemia in neonates, and promote the regression of melanoma. However, the first controlled oncology trials failed to show a significant benefit of BCG therapy in leukemia, lung, breast and colorectal cancer, and enthusiasm for BCG therapy for cancer waned as advances in radio- and chemotherapy were made.

Introduction of BCG for Bladder Cancer Therapy

Only in bladder cancer did clinical trials continue beyond this early period. The Canadian Urologist Alvaro Morales first reported in 1976 on successful outcomes of intravesical BCG in patients with NMIBC [4]. He recognized that the treatment period should last at least 3 weeks due to a delayed type hypersensitivity reaction in the bladder after exposure to BCG. Moreover, since adverse symptoms to treatment resolved after 1 week, he selected a weekly administration schedule. Morales applied intravesical BCG weekly for 6 weeks, which was an arbitrarily chosen regimen that is still used today [4]. He found a significant reduction in the recurrence rate after BCG treatment compared to the period before treatment in nine patients with NMIBC. This regimen was subsequently validated at the University of Texas in San Antonio and at Memorial Sloan-Kettering Cancer Center in New York. Maintenance therapy with repeat intravesical administration of BCG at regular intervals over 36 months was later shown to be important for reducing bladder cancer progression and mortality [5, 6].

BCG: Mechanisms of Action

Immune Response and Cytotoxic Effects

Despite intensive investigations on BCG in bladder cancer, its mechanisms of the antitumor action remain incompletely understood [7]. The pivotal step in BCG efficacy is mycobacterial antigen presentation by phagocytes to CD4+ T cells. After attachment of the intravesical BCG to urothelial and bladder cancer cells via fibronectin and integrin α5β1, BCG is internalized by bladder cancer cells, owing to oncogenic aberrations including constitutively activated macropinocytosis. Following internalization, the antigen processing of phagocytes and the expression of surface antigens on bladder cancer cells (e.g. class II major histocompatibility complex and ICAM-1) are modified. This antigen presentation to CD4+ T cells results in cytokine release (Th1 cytokine profile: IFN-γ, IL-2, IL-12 and TNF-α) and triggers apoptosis of neoplastic cells by natural killer cells, cytotoxic CD8+ cells and neutrophils (Fig. 10.1). Neutrophils are also responsible for BCG treatment-related secretion of TNF-related apoptosis-inducing ligand (TRAIL), a

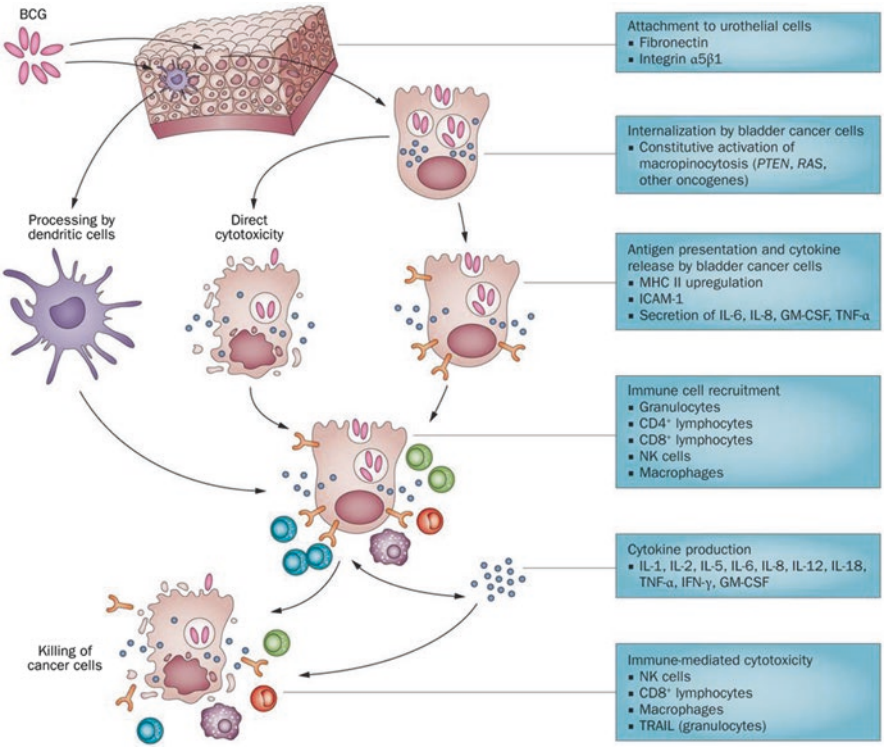

Fig. 10.1 Mechanism of action of intravesical BCG

major contributor to the long term antineoplastic effect. Interferon produced by monocytes induces the expression of TRAIL on the surface of T-cells. These TRAIL-expressing T-cells are responsible for the antitumor defence for weeks to months [8]. Finally, BCG exerts a direct anti-proliferative effect on urothelial cancer cells, by inducing cross-linking of integrins and subsequent cell-cycle arrest and apoptosis [9].

Intravesical BCG: Current Guidelines and Controversies

Indication

Intravesical BCG treatment is indicated in patients with intermediate and high risk NMIBC but not in patients with low risk NMIBC (single low grade Ta tumor <3 cm) [10]. Intermediate risk disease is defined as low grade tumor that is either >3 cm, or multifocal/recurrent, while high risk tumor is defined as any T1 or high grade tumor, including carcinoma in situ.

Induction and Maintenance

Although the true optimal regimen for BCG remains unknown, the original 6 week induction course continues to be used universally [4]. Most of the controversy regarding the therapeutic regimen relates to maintenance therapy. Despite anecdotal claims to the contrary [11], the evidence is clear that maintenance therapy reduces the risk of recurrence and progression [12]. The best data support the use of the full SWOG 8507 regimen, with three weekly doses of BCG at 3, 6, 12, 18, 24, 30 and 36 months after initiation of induction [5, 13]. The European Organization of Research and Treatment of Cancer (EORTC) 30,962 trial comparing one versus 3 years of maintenance and 1/3 dose versus full dose BCG, suggested that full dose BCG over 3 years is better than reduced dose or shortened maintenance in high risk NMIBC, but 1 year of full dose BCG is likely adequate for intermediate risk NMIBC [6].

Variable Efficacy of Different BCG Strains

More than ten different BCG strains are used around the world. These can be categorized into early strains (Russian, Moreau, Tokyo, Sweden and Birkhaug, isolated before 1930) and late strains (Danish, Glaxo, Tice, Connaught, Phipps, Frappier, Prague, RIVM and Pasteur, isolated after 1930). With the present molecular biology techniques these strains have been sequenced completely and genetic variability between strains has been identified. In addition, variations in lipid and glycolipid content of the mycobacterial cell wall have been described. Some of these differences may or may not be related to BCG viability, and efficacy for inhibiting tumor growth and triggering cytokine production [14]. The Connaught and Tice strains are the most widely administered in Europe, and only the Tice strain is currently available in North America. The Connaught strain is also approved for use in North America but it is not available due to lack of production by the manufacturer. Although the different BCG strains are considered to be biosimilar, they may in fact have variable efficacy, as demonstrated in multiple in vitro and in vivo animal models, as well as in clinical trials. In a prospective randomized phase III trial Rentsch et al. demonstrated a stronger immune response and a better recurrence-free survival (RFS) with BCG Connaught compared to BCG Tice (5-year RFS: 74% vs. 48%) [15]. This trial was limited by a lack of maintenance BCG. Subsequent retrospective reviews failed to confirm this difference in strains if maintenance therapy is used [16, 17]. The efficacy of BCG Tice was also inferior to BCG RIVM in an older study [18] (5-year RFS: 36% vs. 54%), even though this study was underpowered to detect statistically significant outcomes. The field still lacks convincing prospective validation studies to permit a final conclusion regarding the differential efficacy of available BCG strains. One large phase III trial is currently underway to compare BCG Tice to BCG Tokyo strain, the results of which could pave the way for regulatory approval of BCG Tokyo in North America if successful [19].

Another critical unmet need in this context is a biomarker to guide patient selection for intravesical BCG therapy, and to predict response to treatment. Various

biomarkers (e.g. cytokine panels, gene signatures, methylation status, IL-2 gene expression, urinary IL-8 and IL-18) have been investigated in order to predict efficacy of BCG treatment [20–24]. One with potential clinical utility is multi-color fluorescent in situ hybridization (FISH) [25], although none of these markers have been adopted in routine clinical practice.

BCG Versus Intravesical Chemotherapy

Several randomized controlled trials have confirmed the superiority of BCG over intravesical chemotherapy in the prevention of tumor recurrence in patients with intermediate and high risk NMIBC. Two meta-analyses comparing BCG to intravesical chemotherapy showed that BCG more effectively reduced the risk of recurrence, and only BCG with maintenance therapy was able to decrease the risk of progression [13, 26]. A limitation of these studies is the use of only induction chemotherapy without maintenance and the absence of mitomycin optimization (e.g. increased drug concentration and urine alkalinisation) [27]. Compared to transurethral resection of the bladder (TURB) alone, recurrences are decreased by 30% with the addition of BCG maintenance and by 20% with the addition of mitomycin maintenance. Although the efficacy of BCG is believed to be superior to intravesical chemotherapy, it is associated with a higher rate of side effects. For this reason, and because the risk of progression is relatively low in intermediate disease intravesical chemotherapy remains a therapeutic option in patients with intermediate risk NMIBC.

Toxicity and Side Effects

Side effects can be classified as local or systemic (Table 10.1). Serious side effects are encountered in <5% of patients and can be treated effectively in almost all cases [28, 29]. In the original SWOG 8507 trial of maintenance therapy, only 16% of patients completed 36 months of therapy due to either disease recurrence/progression or side effects. More recent trials have reported higher compliance rates, with approximately one quarter of patients delaying treatment and 20% discontinuing treatment altogether due to side effects. As a result, several secondary measures have been investigated to reduce toxicity. To prevent adverse effects, BCG should not be administered during the first 2 weeks after TURB, in patients with gross hematuria, after traumatic catheterization and with symptomatic urinary tract infection. However, the presence of leukocyturia, microscopic hematuria or asymptomatic bacteriuria is not a contraindication to BCG administration. The recent EORTC trial demonstrated that dose reduction to 1/3 dose BCG did not reduce toxicity, so that this measure can be considered ineffective [6]. The concomitant use of antibiotics can reduce toxicity [30], but longer follow-up and larger patient cohorts would be necessary to demonstrate that there is no detrimental effect on treatment efficacy [31].

Table 10.1 Local and systemic adverse effects associated with intravesical BCG therapy

Adverse effects	Rate (%)	Effect on delivery	Management	Recommendation to postpone treatment
Local				
BCG-induced cystitis	47	6% delay, 12% stop	NSAID	No, unless symptoms persist or worsen
Bacterial cystitis	26	6% delay, 2% stop	Urine culture and empiric antibiotics	Resume when cystitis resolved
Visible hematuria	35		Urine culture; cystoscopy if persistent	Resume when hematuria resolved
Systemic				
Fever ≥39 °C	15	5% stop	If persistent, order urine/blood culture, chest X-ray; treat with ≥2 antimicrobial agents[a]; infectious disease consultation	Discontinue BCG therapy permanently if >48 h duration
General malaise	23	3% stop	Resolves within 48 h with or without NSAID/acetaminophen	
Arthralgia and/or arthritis	<1		NSAID	
BCG sepsis	<1		Combination of high-dose antimicrobial agents[a]; systemic corticosteroids	Discontinue BCG therapy permanently
Allergic reaction	3	1% stop	Antihistamines and anti-inflammatory agents	Delay therapy until reactions resolve

Van der Meijden et al. [28], EAU Guidelines on NMIBC [17], Gontero et al. [45], Mathes et al. [29]
[a]Including fluoroquinolone/isoniazid/rifampin

BCG Failure

Several meta-analyses have confirmed that BCG after TURB is superior to TURB alone. Regardless of patient risk category, recurrences were reduced by a quarter with the combination of TURB and BCG induction when compared to TURB alone. This rate can be increased to a third with maintenance therapy. The absolute risk reduction in progression in the Sylvester meta-analysis of BCG therapy was 4% (13.8% without BCG versus 9.8% with BCG) after a median follow-up of 2.5 years [26].

Several common definitions of BCG failure are defined in Table 10.2 [32, 33]. The management of patients who have failed BCG therapy is complex [34]. The risk of progression increases with each subsequent course of intravesical therapy, and no therapy has more than an approximately 20% recurrence-free survival rate at 2 years [35]. Therefore, radical cystectomy is generally recommended for high risk disease that has failed BCG. Patients relapsing more than 12 months after prior BCG therapy can be re-challenged with intravesical BCG with reasonable results [36].

Table 10.2 Common definitions of BCG failure

Groups of BCG failure	Definition
Resistant	Recurrence or persistence of lesser stage or grade urothelial carcinoma after induction BCG at 3 months that is no longer present after additional BCG (re-induction or first maintenance) at 6 months, with or without TUR
Refractory	Failure to achieve a disease-free state by 6 months after initial BCG therapy with either maintenance or re-induction at 3 months Includes any progression in stage, grade, or disease extent after induction BCG at 3 months
Intolerant	Recurrence after a less-than-adequate course of therapy due to a serious adverse event or symptomatic intolerance that mandates discontinuation of further BCG
Relapsing	Recurrence of disease after achieving a disease-free status at 6 months Early (within 12 months), intermediate (12–24 months), or late (24 months)
Unresponsive	One of the following: 1. Recurrent high grade Ta or CIS after induction BCG plus one round of maintenance BCG, or a second round of induction BCG 2. Recurrent high grade T1 after induction BCG 3. Early BCG relapse defined by recurrent high grade Ta/T1 within 6 months of last dose of BCG after prior response, or recurrent CIS within 12 months of last dose of BCG after prior response

Nieder et al. [32]
https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM529600.pdf

If bladder preservation is sought in BCG refractory and unresponsive disease, several strategies are available [37]. BCG can be combined with interferon-alpha, although there is no evidence that this is any more efficacious than repeat BCG alone [38]. Small phase II trials suggest that intravesical gemcitabine and docetaxel are well-tolerated alternatives for BCG failure with modest but measurable activity. Electromotive delivery of mitomycin alternating with BCG over 12 months proved superior to BCG alone in a prospective randomized clinical trial involving patients with BCG-naïve, high-risk NMIBC, but this has not been adequately studied in patients recurring after BCG [39]. Intravesical chemohyperthermia is similarly an option [40]. Due to the low response rates and the poor durability of those responses, radical cystectomy remains the standard of care for patients failing intravesical BCG therapy.

Future Directions

Ongoing Trials

Ongoing trials investigating BCG treatment and combination therapies as well as treatment options after BCG failure are summarized in Table 10.3. BCG is one of the most studied medicines in Urology. Although we have learned much about this treatment, many questions regarding its use remain.

Table 10.3 Active or recently completed clinical trials registered on Clincal.Trials.gov involving intravesical BCG therapy for bladder cancer, or novel therapies for disease recurrence after BCG therapy (accessed Nov. 27, 2018)

NCT number	Title	Experimental agent	Design	Primary outcome measures	Completion date
BCG combination trials					
NCT00794950	Bacillus Calmette-Guerin Followed by Sunitinib for the Treatment of High Risk Non-muscle Invasive Lower Urinary Tract Urothelial Carcinoma	Sunitinib: Multi-targeted receptor tyrosine kinase inhibitor	Phase 2	Complete response rate at 3 months	December 2018
NCT02753309	A Study of Rapamycin Combined With Intravesical BCG in Patients with Non-muscle Invasive Bladder Cancer	Rapamycin +/− BCG	Randomized phase 2	Pharmacodynamic immunologic endpoints	December 2018
NCT02138734	A Study of Intravesical Bacillus Calmette-Guerin (BCG) in Combination with ALT-803 in Patients with BCG-naive Non-muscle Invasive Bladder Cancer	ALT-803: Mutated IL-15 analogue combined with IL-15Rα-Fc fusion	Phase 1/2	Safety Time to recurrence	October 2019
NCT03672240	Study of APL-1202 in Non-Muscle Invasive Bladder Cancer Patients Who Are Resistant to One Induction Course of BCG Treatment (NMIBC)	Oral methionine aminopeptidase type II (MetAP2) inhibitor	Phase I	Safety	October 2019
NCT03022825	QUILT-3.032 A Multicenter Clinical Trial of Intravesical Bacillus Calmette-Guerin (BCG) in Combination with ALT-803 in Patients With BCG Unresponsive High Grade Non-muscle Invasive Bladder Cancer	ALT-803: Mutated IL-15 analogue combined with IL-15Rα-Fc fusion	Phase 2	Complete response (24 months)	September 2020
NCT02948543	Adding Mitomycin C to Bacillus of Calmette-Guerin (BCG) as Adjuvant Intravesical Therapy for High-risk, Non-Muscle-invasive Bladder Cancer (BCG + MMC)	Combination BCG + Mitomycin	Randomized phase 3	Disease free survival (up to 5 years)	December 2020
NCT02792192	Safety and Pharmacology Study of Atezolizumab Alone and in Combination with Bacille Calmette-Guérin (BCG) in High-Risk Non-muscle-Invasive Bladder Cancer (NMIBC) Participants	Atezolizumab +/− BCG	Phase 1b/2	1b: Safety 2: CR (6 months)	November 2021

NCT03317158	ADAPT-BLADDER: Modern Immunotherapy in BCG-Relapsing Urothelial Carcinoma of the Bladder	Durvalumab +/− BCG Durvalumab +/− radiation	Phase 1/2	1: Safety 2: Recurrence free survival (6 months)	September 2021
NCT03519256	A Study of Nivolumab or Nivolumab Plus Experimental Medication BMS-986205 with or Without Bacillus Calumette-Guerin (BCG) in BCG Unresponsive Bladder Cancer That Has Not Invaded Into the Muscle Wall of the Bladder (CheckMate 9UT)	Nivolumab +/− BMS-986205 (IDO inhibitor) +/− BCG	Randomized phase 2	Complete response and duration of CR in patients with CIS; event free survival for non-CIS	April 2023
NCT03528694	Assessment of Efficacy and Safety of Durvalumab Plus BCG Compared to the Standard Therapy with BCG in Non-muscle Invasive Bladder Cancer (POTOMAC)	Durvalumab	Randomized phase 3	Disease free survival (up to 4 years)	November 2024
NCT03711032	Efficacy and Safety of Pembrolizumab (MK-3475) in Combination With Bacillus Calmette-Guerin (BCG) in High-Risk Non-muscle Invasive Bladder Cancer (HR NMIBC) (MK-3475-676/KEYNOTE-676)	Pembrolizumab	Randomized phase 3	Complete response of patients with CIS	November 2024
NCT03664869	Electromotive Mitomycin-C (EMDA-MMC) in Preventing Recurrences in High-risk Non-muscle-invasive Bladder Cancer (FB10)	Electromotive Mitomycin	Randomized phase 3	Recurrence	November 2025
BCG failure trials					
NCT02015104	Study of Bacillus Calmette-Guerin (BCG) Combined with PANVAC Versus BCG Alone in Adults With High Grade Non-muscle Invasive Bladder Cancer Who Failed At Least 1 Course of BCG	Poxviral-based vaccine therapy targeting CEA and MUC1 in carcinoma)	Randomized, phase 2	Disease-free survival	Accrual complete

(continued)

Table 10.3 (continued)

NCT number	Title	Experimental agent	Design	Primary outcome measures	Completion date
NCT01259063	RAD001 and Intravesical Gemcitabine in BCG-Refractory Primary or Secondary Carcinoma In Situ of the Bladder	RAD001 (Everolimus): mTOR inhibitor	Phase 1/2	1: Safety 2: Disease free at 1 year	Accrual complete
NCT01732107	Dovitinib in BCG Refractory Urothelial Carcinoma With FGFR3 Mutations or Over-expression	Multi-tyrosine kinase inhibitor (especially FGFR and VEGFR)	Phase 2	Complete response rate at 6 months	Accrual complete
NCT01625260	A Study of ALT-801 in Patients with Bacillus Calmette-Guerin (BCG) Failure Non-muscle Invasive Bladder Cancer	Recombinant protein consisting of IL-2 fused to a humanized soluble T-cell receptor directed against a p53-derived antigen	Phase 1/2	Safety	Accrual complete
NCT02009332	Phase 1/2 Study of ABI-009 in BCG Refractory or Recurrent Nonmuscle Invasive Bladder Cancer	Nanoparticle albumin-bound rapamycin (mTOR inhibitor)	Phase 1/2	Safety	December 2020
NCT02773849	A Study to Evaluate INSTILADRIN® in Patients With High-Grade, Bacillus Calmette-Guerin (BCG) Unresponsive NMIBC	Recombinant interferon alpha2b in adenovirus vector with concomitant excipient Syn3	Phase 3 (single arm)	Complete response in patients with CIS	June 2022
NCT02449239	Vicinium Treatment for Subjects with Non-muscle Invasive Bladder Cancer Previously Treated with BCG	Recombinant fusion protein expressing humanized antibody fragment specific for the epithelial cell adhesion molecule (EpCAM) linked to the toxin ETA(252–608)	Phase 3 (single arm)	Complete response in patients with CIS	March 2019

NCT02365818	Safety and Efficacy of CG0070 Oncolytic Virus Regimen for High Grade NMIBC After BCG Failure (BOND2)	Oncolytic virus expressing GMCSF	Phase 2	Durable complete response	December 2019
NCT02844816	Atezolizumab in Treating Patients With Recurrent BCG-Unresponsive Non-muscle Invasive Bladder Cancer	Atezolizumab	Phase 2	Complete response at 6 months in CIS patients and event free survival at 18 months in all patients	February 2020
NCT02808143	Pembrolizumab and BCG Solution in Treating Patients With Recurrent Non-Muscle-Invasive Bladder Cancer	Intravesical pembrolizumab	Phase 1	Safety	February 2020
NCT02202772	Intravesical Cabazitaxel, Gemcitabine, and Cisplatin (CGC) in the Treatment Urothelial Carcinoma of the Bladder (CGC)	Cabazitaxel, gemcitabine, and cisplatin	Phase 1	Safety	December 2020
NCT03421236	Intravesical Ty21a for the Treatment of Patients With Non-muscle-invasive Bladder Cancer (NMIBC)	Ty21a (attenuated bacterial vaccine)	Phase 1	Safety	March 2021
NCT02982395	Study to Evaluate the Efficacy and Safety Of Intravesical Nanoxel®M In BCG Refractory NMIBC	Nanoxel®M (nanoparticle docetaxel) vs. Mitomycin	Phase 3	Recurrence free at 1 year	June 2021
NCT03258593	Durvalumab and Vicinium in Subjects With High-Grade Non-Muscle-Invasive Bladder Cancer Previously Treated With Bacillus Calmette-Guerin (BCG)	Durvalumab Vicinium	Phase 1	Safety	July 2021
NCT03552796	sEphB4-HSA in Treating Participants With BCG-Unresponsive or Refractory Bladder Cancer	Recombinant EphB4-HSA fusion protein	Phase 1	Safety	October 2021
NCT03719300	Codex: Study of BC-819 in NMIBC Patients Unresponsive to BCG (Codex)	BC-819: Recombinant DNA plasmid that directs the expression of diphtheria toxin specifically in presence of H19 transcription factor	Phase 2	Complete response in patients with CIS	November 2021

(continued)

Table 10.3 (continued)

NCT number	Title	Experimental agent	Design	Primary outcome measures	Completion date
NCT02901548	Phase 2 Durvalumab (Medi4736) for Bacillus Calmette-Guérin (BCG) Refactory Urothelial Carcinoma in Situ of the Bladder	Durvalumab	Phase 2	Complete response at 6 months	December 2021
NCT02625961	Study of Pembrolizumab in Participants With High Risk Non-muscle Invasive Bladder Cancer (KEYNOTE-057)	Pembrolizumab	Phase 2	Complete response Disease free survival	July 2023
NCT03335059	Mitomycin C Intravesical Chemotherapy in Conjunction With Synergo® Radiofrequency-Induced Hyperthermia for Treatment of Carcinoma in Situ Non-Muscle Invasive Bladder Cancer Patients Unresponsive to Bacillus Calmette-Guérin, With or Without Papillary Tumors. (RITE-USA)	Chemohyperthermia with Mitomycin	Phase 2	Complete response at 3 months	December 2024
BCG trials					
NCT01082510	Study of the Efficacy of Maintenance Therapy Using Uracil-tegafur (UFT) or Bacille Calmette-Guerin (BCG) for the Prevention of Recurrences of Superficial Bladder Cancer (EMBARK Study)	UFT: containing uracil and tegafur (prodrug of 5-fluorouracil)	Randomized phase 3	Recurrence-free survival (36 months)	December 2019
NCT03091660	S1602: Different Strains of BCG With or Without Vaccine in High Grade Non- Muscle Invasive Bladder Cancer	Intradermal BCG vaccine BCG Tokyo strain	Randomized phase 3	Time to high grade recurrence	February 2025

Vaccination

Because the beneficial effects of BCG rely on the immune system, previously BCG-sensitized patients are thought to respond better to intravesical BCG. In one trial, simultaneous intradermal BCG application at the time of the intravesical BCG induction course did not improve the therapeutic benefit [41]. A longer exposure to the pathogen seems to be necessary, as the immune stimulation generally peaks at 3 weeks and persists for 6 months. This ¨vaccination¨ effect might be a possible mechanistic explanation for the additional benefit of maintenance therapy over induction therapy alone [42]. A new cooperative group trial testing BCG vaccination in patients with BCG-naïve high risk NMIBC is currently ongoing in the US (¨Prime Trial¨) [19].

Modulating BCG

BCG retains many features of pathogenic mycobacteria that counteract the potential immunologic benefits of treatment. In particular, BCG inhibits macrophage apoptosis. Manipulating BCG strains to induce apoptosis would restrict cancer cell proliferation and promote efficient presentation of tumor antigens by infected cells [43]. In addition, tumor antigens specific to various tumor types have been discovered recently. It is therefore conceivable that BCG modified to carry bladder cancer antigens could augment the anti-tumor immune response. In addition, enhanced internalization of BCG into bladder cancer cells could increase the anti-tumor effect. For example, bladder cancer cells that produce human β-defensin-2 (HBD2) are protected against BCG, and Kim et al. were able to show that blocking this defence with an anti-HBD2 antibody increased the internalization and effectiveness of BCG [44]. However, these options have only been investigated in *in vitro* and *in vivo* models without clinical experience.

Conclusions

Although introduced more than 40 years ago, BCG remains the standard treatment in patients with NMIBC. Intravesical BCG treatment is indicated as first line therapy in most patients with high risk NMIBC (high grade tumors, T1 tumors and CIS) and is also frequently used in intermediate risk NMIBC. Maintenance therapy for up to 3 years reduces recurrence and progression. Mild to moderate side effects are common but can generally be managed effectively without treatment discontinuation, and severe side effects are rare. Meticulous patient follow-up is required to detect bladder cancer progression despite optimal BCG therapy.

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The Microbiome in Female Urology

11

Whitney Smith and Alana Murphy

Abbreviations

BPS	Bladder pain syndrome
EQUC	Expanded quantitative urine culture
HMP	Human microbiome project
IC	Interstitial cystitis
NIH	National Institute of Health
OAB	Overactive bladder
qPCR	Quantitative polymerase chain reaction
SUI	Stress urinary incontinence
UUI	Urgency urinary incontinence

Background

All human beings share a largely symbiotic and sometimes pathologic relationship with a variety of microbial species and strains. Although there are similarities across all humans, an individual's unique microbiome can be thought of as a distinct fingerprint. Even in healthy individuals, the microbial environment can vary from individual to individual. While *microbiota* is defined as the "microbial taxa associated

W. Smith
Wake Forest University, Winston-Salem, NC, USA
e-mail: whsmith@wakehealth.edu

A. Murphy (✉)
Department of Urology, Thomas Jefferson University/Thomas Jefferson University Hospital, Philadelphia, PA, USA
e-mail: alana.murphy@jefferson.edu

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with humans," *microbiome* is defined as the "catalog of these microbes and their genes" [1]. The human microbiome is of great interest to researchers and clinicians, as the balance between symbiotic and pathologic microbes has been linked to different disease states.

Much of our knowledge regarding the human microbiome is derived from the Human Microbiome Project (HMP), a study funded by the National Institute of Health (NIH) and conducted between 2008 and 2013. The study collected samples from hundreds of healthy study participants from five different sites on the human body: nasal passages, oral cavity, skin, gastrointestinal tract, and genitourinary tract [2]. Study of the vaginal microbiome revealed a low level of diversity among bacterial species and the predominant organism was *Lactobacillus*. Although this study did not assess the microbiome in human urine samples, research has emerged linking the genitourinary tract microbiome to common urologic disorders. Characterization of both normal and abnormal microbiomes has the potential to change the clinical assessment of and treatment for common bladder syndromes.

When examining the current female microbiome literature and thinking about future research in the field, it is important to take note of how human specimens are collected and cultured. The vast majority of clinicians continue to rely on mid-stream voided urine samples and standard bacterial culture techniques to identify bacteria in the bladder. In 2019, this remains the gold standard for management of urinary tract infection patients. However, standard urine collection and culture techniques are not optimal for identifying non-traditional pathologic bacteria that may be affecting the lower urinary tract. The current standard of care does not lend itself to detecting bacteria that are slow growing, oxygen intolerant, present in small quantities, or require a special medium for growth [3].

In response to the shortcomings of traditional urine culture techniques, several studies that examined the urinary microbiome have used culture independent techniques, which are more sensitive. The most commonly used culture independent technique in the literature is gene sequencing using 16s ribosomal RNA. This technique was used in the initial discovery of the female microbiome by Wolfe and colleagues in 2012 [4]. Both the research community and clinicians also recognize the impact of specimen collection. Voided urine samples, even mid-stream samples, are subject to vulvar contamination. Within the female microbiome research community, catheterized specimens are becoming the gold standard.

Female Bladder Microbiome

Historically, the lower urinary tract was thought to be a sterile environment. However, current and on-going research regarding the female microbiome has challenged this theory of bladder sterility. In a study conducted by Wolfe and colleagues, urine from women without bladder symptoms undergoing surgery for benign gynecological indications was considered a control group. Their urine samples were compared to women undergoing surgeries for pelvic organ prolapse or incontinence with at least one bladder symptom [4]. Urine samples were collected via a voided

clean catch technique, transurethral catheterization, and/or suprapubic aspiration. The urine specimens were analyzed for bacteria using standard bacterial culture, light microscopy, and 16s ribosomal gene sequencing. Even in women without bladder symptoms, it was common to have bacteria detected using light microscopy and 16s ribosomal sequencing. The voided urine specimens often contained a mix of bladder and genital flora contamination, while transurethral catheterization specimens and suprapubic aspiration specimens reflected a similar array of lower urinary tract flora. If the lower urinary tract is not a sterile environment, then we are left to determine the role of bladder microbiota and its relationship to common bladder syndromes.

Urgency Urinary Incontinence

Urgency is the sudden desire to pass urine which is difficult to defer and urgency urinary incontinence (UUI) is defined as involuntary leakage of urine associated with urgency [5]. Urgency is the hallmark of Overactive Bladder syndrome (OAB). Patients with OAB may or may not experience episodes of UUI. OAB is a common condition affecting approximately 17% of women over 44 years of age [6, 7]. The etiology of most OAB cases is thought to be idiopathic. Since a urinary tract infection and OAB are both associated with urgency and frequent urination, a clinician is required to rule out the possibility of a urinary tract infection when diagnosing a patient with OAB [8]. Due to the overlapping symptomatology, it has been hypothesized that the bladder microbiome may play a role in the development of OAB and UUI.

In a study by Pearce and colleagues, transurethral catheterized urine specimens were obtained from 60 women with UUI and 58 women without complaint of UUI [9]. 16s rRNA sequencing was used to classify bacteria and live bacteria were isolated using expanded quantitative urine culture (EQUC). In comparison to standard urine culture technique, EQUC increases the inoculated urine volumes, utilizes an array of media types, and incubates specimens in more environments at different temperatures. A total of 64 of the 71 urine specimens deemed "no growth" by standard urine culture technique grew bacteria using the EQUC method. In women with and without UUI, the majority of the cultured bacteria were from the *Lactobacillus* and *Gardnerella* genera. In the UUI group, *Gardnerella* and *Aerococcus* were detected more commonly compared with the control group by 16s RNA sequencing. Compared to the control group, the following were found more frequently in the UUI group: *Actinobaculum*, *Actinomyces*, *Aerococcus*, *Arthrobacter*, *Corynebacterium*, *Gardnerella*, *Oligella*, Staphylococcus, and Streptococcus. Within the *Lactobacillus* species, differences were observed between women with and without UUI. *Lactobacillus gasseri* was more commonly detected in the UUI group and *Lactobacillus crispatus* was more frequently detected in the control group.

In a separate study by Brubaker and colleagues, women with UUI who were enrolled in the Anticholinergic Versus Botulinum Toxin A Comparison (ABC) trial were randomized to receive intradetrusor onabotulinum toxin A and oral placebo or cystoscopic placebo injection and active oral medication [10]. Urine samples were

collected by transurethral catheterization and analyzed by quantitative polymerase chain reaction (qPCR). Of the 155 participants, qPCR detected bacteria in 38.7% of the samples. Patients found to have bacteria using qPCR had more severe UUI, with a higher baseline number of UUI episodes per day compared to those who had no bacteria detected [5.71(±2.6) versus 4.72(±2.86), p = 0.0045].

Finally, a recent study conducted by Karstens and colleagues also sought to characterize the bladder microbiome in women with and without UUI [11]. Transurethral catheterized urine samples were obtained from a total of 20 women with and without UUI. Bacterial isolation was performed using qPCR techniques. Bacteria were isolated from both groups, which lends support for the hypothesis that the lower urinary tract is not sterile even in the absence of bothersome urinary symptoms. Although the microbiome distribution differed between the two groups, it is hard to draw conclusions regarding the clinical significance of this finding. Interestingly, the study found that UUI severity scores were inversely related to biodiversity of the bladder microbiome. This finding indicates that biodiversity can play a protective role against UUI.

The current literature suggests that the bladder microbiome may play a role in the pathophysiology of UUI and may hold potential for future treatment strategies. One of the challenges in treating overactive bladder are managing patients who are unresponsive to oral therapies for UUI. In women undergoing treatment of UUI with solifenacin, non-responders have been found to have a less diverse microbiome compared to clinical responders [12]. Such a trend provides additional evidence that a diverse bladder microbiome may have a protective role in the development of bothersome UUI. Future research will help elucidate how manipulation of the bladder microbiome can help alleviate UUI severity and improve overall OAB symptomatology.

Stress Urinary Incontinence

Stress urinary incontinence (SUI) is the involuntary loss of urine during physical exertion [5]. SUI is another common chronic urologic condition with prevalence rates ranging from 20% to 40% in the United States [6]. As part of the Value of Urodynamic Evaluation Study, which was a multicenter, randomized clinical trial comparing urodynamics to office based evaluation as part of the preoperative assessment for SUI, urine samples were obtained either by transurethral catheterization or by voided specimens [13]. The presence of bacteria was assessed using 16s rDNA sequencing. Of the specimens obtained, the most common organisms isolated included *Lactobacillus*, *Gardnerella*, *Streptococcus*, *Corynebacterium*, or no dominant species. The study found that women with SUI undergoing surgery who had increased microbial diversity also had increased UUI severity. Microbial diversity did not correlate with SUI symptoms. This finding contradicts the previously mentioned studies that examined the link between bladder microbiome diversity and UUI severity.

Defining the role of the female microbiome in SUI is in its infancy. At this time there is minimal data in the literature linking SUI and the bladder microbiome.

Interstitial Cystitis/Bladder Pain Syndrome

Interstitial cystitis (IC), also referred to as bladder pain syndrome (BPS), is a chronic condition that is often difficult to diagnose and treat. It is characterized by chronic bladder pain and or pressure that is often exacerbated by bladder filling and one other lower urinary tract symptom (often urinary frequency) [14]. It is a diagnosis of exclusion. Many etiologies for IC/BPS have been proposed, including infection, autoimmune disorder, and mucosal damage with resulting c-fiber and mast cell activation and downstream histamine release. Ongoing research is starting to explore whether or not the female microbiome plays a role in symptomatic flares in IC/PBS.

Abernethy and colleagues sought to investigate differences in the urinary microbiome and cytokine levels between women with and without IC/BPS. These women included those diagnosed with IC/BPS matched with women without IC/BPS. To analyze for bacteria, catheterized urine samples were analyzed using ribosomal RNA sequencing. Compared to controls, the IC/BPS group was less likely to contain *Lactobacillus acidophilus* (*L. acidophilus* was associated with a less severe symptom scores) and contained a less diverse microbiome. The IC/PBS specimens also contained higher levels of the pro-inflammatory cytokines, specifically macrophage-derived chemokine and interleukin-4 [15].

Although Nickel et al. showed no difference in ICS/BPS flare episodes in women who had bacteriuria and those that did not, the samples collected in this study were collected as midstream urine cultures and standard culture methods were used [16]. In a MAPP Network Study, initial stream and midstream urine specimens were analyzed using an Ibis T-5000 Universal Biosensor system, which is a culture independent technique. They found that fungal species were more likely to be present in the midstream urine specimens in women who reported a flare of IC/BPS symptoms [17].

These studies show the potential importance of the bladder microbiome in the symptomatology of IC/BPS and, perhaps, a step forward in understanding a potential etiology for this challenging condition.

Conclusion

The study of the female microbiome as it relates to the common bladder conditions in female urology is still in its infancy. However, this field is rapidly expanding and there are a host of opportunities for future research. To date, the most promising applications are with UUI and IC/BPS. There have been some links to stress urinary incontinence, but, at this time, more information is needed. Characterization of the female bladder microbiome has the potential to shape future clinical assessment and treatment algorithms for UUI and IC/BPS patients.

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The Microbiome in the Prostate: Prostatitis and Prostate Cancer

12

Claudia Chavez-Munoz, Bhavish Kowlessur, and Alan So

Introduction

The development of novel RNA and DNA sequencing techniques have advanced the study of the microbiota in genitourinary organs. Once thought as a "sterile" organ with occasional involvement of bacteria and viruses, recent genomic studies have revealed that the interaction between microbes and the prostate is much more complex. The study of the microbiome of the prostate, defined as the collection of genomes, genes and products of the microbes present in the prostate, has only started the exploration of the role and impact of the microbial environment on prostate health, function, and pathology [1–3]. As the prostate is located near the rectal wall, an altered enteric microbiome and changes in intestinal permeability may lead to changes within the prostate that may be associated with the inducement of different pathological conditions, including inflammation and neoplasia.

There is evidence that different microbiomes (cutaneous, oral, gastric, gut, colon) have direct relationship to the initiation or progression of different diseases [4–11]. For example, it has been shown that deviations from the normal human gut microbiome have been discovered in a variety of diseases and conditions, including inflammatory bowel disease, colorectal cancer, obesity/metabolic syndrome, type 2 diabetes mellitus, breast cancer, some autoimmune diseases, autism spectrum disorder, post-traumatic stress disorder and responsiveness to visceral pain. Some studies have revealed even more relationships between the gut microbiome and the central

Claudia Chavez-Munoz and Bhavish Kowlessur contributed equally to this work.

C. Chavez-Munoz · B. Kowlessur · A. So (✉)
Department of Urologic Sciences and Vancouver Prostate Centre, University of British Columbia, Vancouver, BC, Canada
e-mail: cchavez@prostatecentre.com; bkowlessur@prostatecentre.com; alan.so@ubc.ca

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nervous system (CNS), suggesting the existence of a "gut-brain axis" where the gut microbiome modulates the CNS and or vice versa [12–15].

With the growth in the knowledge of the prostate microbiome, the challenge of establishing causal relationships between the microbiome and health and disease increases. The Human microbiome project was established in 2008 with the aim of developing a comprehensive characterization of the human microbiome and analysis of its role in human health and disease and has increased the knowledge of microbiome that exists in the prostate [16]. These studies show the incredible diversity of the microbes that co-exist in the prostate. As well, the microbiome can change during a patient's life, seasonally, or with environmental changes (infection, treatment, diet, hormone state or lifestyle) [1–3, 17–29]. In this chapter, recent studies of the microbiome of the prostate will be described as well as the potential role of the microbial environment in prostate inflammation or development of malignancy.

The Prostate Microbiome and Prostatitis

Several etiological factors are proposed to contribute to the development of prostate inflammation: bacterial and viral infections, dietary carcinogenic compounds, alterations in testosterone to estradiol ratio, physical trauma caused by the presence of corpora amylacea and prostatic calculi and urine reflux [30].

Prostatitis is classified, according to the National Institutes of Health (Table 12.1).

Category I prostatitis has been thought to be caused by an array of organisms: primarily gram negative frequently by species such as *E. coli* (65–80% of infections). However approximately 10% are gram positive organisms such as Enterococcus species (5–10% of infections) [31].

Table 12.1 Classification system for prostatitis by the National Institutes of Health (NIH)

Category	Description
I	Acute infection of the prostate gland
II	Chronic infection of the prostate gland
III (Chronic pelvic pain syndrome)	Chronic genitourinary pain in the absence of uropathogenic bacteria localized to the prostate gland employing standard methodology
IIIA (Inflammatory chronic pelvic pain syndrome)	Significant number of white blood cells in expressed prostatic secretions, post prostatic massage urine sediment or semen
IIIB (Non-inflammatory chronic pelvic pain syndrome)	Insignificant number of white blood cells in expressed prostatic secretions, post prostatic massage urine sediment or semen
Asymptomatic inflammatory prostatitis	White blood cells (and/or bacteria) in expressed prostatic secretions, post prostatic massage urine sediment or semen or histologic specimens of prostate gland

The Microbiome in Urine

It used to be clinical dogmatic thinking that the urinary tract is sterile; however, recent genomic evidence however suggests that there is bacteria within the urinary tract that was previously not detectable in traditional culture based assays. Hilt et al. hypothesised that standard culture methods are inadequate and in fact only identify aerobic fast growing bacteria [32]. Their team devised an expanded quantitative culture protocol and tested it in women with Overactive Bladder as well as a control group without evidence of urinary disease. Their results demonstrate that 80% of the overall study population had culturable bacteria.

Culture-dependent and culture-independent studies reveal a consensus for the female urinary microbiome that includes Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, and Fusobacteria at the phyla level and Lactobobacillus, Corynebacterium, Streptococcus, Actinomycese, Staphylococcous, Gardnerella, Micrococcus, Bifidobacterium, Prevotella, Enterococcus, Comamodacea, and Lachnospiracea in the genus level [32–35].

The male urinary microbiome is less well studied than its female counterpart both in the context of its composition and association with disease. The emerging consensus for the male urinary microbiome is that it is predominated by the presence of Corynebacterium, Staphylococcus, Streptococcus, Anaerococcous, Finegoldia, Lactobacillus, Peptoniphilus, Enterobacteriaceae, Pseudomonas, Actinobaculum, Gammaproteobacter, Actinomyces, and Gardnerella [35–37] at the genus levels with Corynebacterium, Staphylococcus, and Streptococcus as the three dominant genera.

Another study showed that the urinary flora in men with detectable but asymptomatic sexual transmitted infections (STI) were different from men without detectable STI. Therefore, the study concluded that the composition of the urinary microbiome may influence susceptibility to infection with STI such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* [38].

Other studies have investigated clinical associations between urinary microbiome and different urgency urinary incontinence treatments. Brubaker and collaborators analyzed bacterial DNA in catheterized urine from women with urgency urinary incontinence and concluded that the urinary microbiome contributed to the urgency urinary incontinence episodes, symptoms severity and post-treatment urinary tract infection risk [39]. On the other hand, Pearce and colleagues found a lower number of Lactobacillus sequences in women who experienced a post-treatment urinary tract infection compared to those without urinary tract infections [40]. They concluded that urinary bacterial DNA was associated with treatment response and concurred with the findings of Brubaker [39] regarding the implications of bacterial DNA in urgency urinary incontinence episodes and post-treatment urinary tract infections. Thomas-White and collaborators investigated the relation between urinary microbiome characteristics and the clinical response to solifenacin, an oral medication for urgency urinary incontinence [41]. The authors found that the

response to solifenacin was better in women with lower bacterial abundance and bacterial diversity. They concluded that an individual's urinary microbiota could be related to urgency urinary incontinence status and treatment response. They also suggested that if there was a microbial signature associated with treatment response, it would be interesting to phenotype the urinary microbiota of urgency urinary incontinence-affected women before treatment decisions.

The Urinary Microbiome and Inflammatory Diseases

Several studies have investigated the role of urinary microbiota in relation to interstitial cystitis, neurogenic bladder dysfunction, sexually transmitted infections and chronic prostatitis/chronic pelvic pain syndrome. In fact, it has been shown that women with interstitial cystitis showed clear differences in the taxonomic composition, richness and diversity compared to the microbial profile for asymptomatic healthy individuals [42]. The study showed an increase in Lactobacillus genus and a decrease in overall richness and ecological diversity in women with interstitial cystitis. Lactobacillus is well-known to be part of the normal vaginal microflora maintaining an acidic environment protecting it against infections [43, 44]. However, there are studies indicating the association of specific Lactobacillus (*L. delbrueckii* and *L. gasseri*) with urinary tract infection and urgency urinary incontinence [40, 45].

Another study analyzed the urinary microbiome from patients with neurogenic bladder dysfunction, finding a significant enrichment of Klebsiella, Enterococcus and Escherichia bacteria as opposed to healthy controls showing enrichment of Lactobacillus and Corynebacterium [46]. Nelson and colleagues analyzed the urinary microbiome from men with sexually transmitted infections, finding that the microbiome was clearly dominated by bacteria genera that do not grow under standard culture conditions such as Sneathia, Gemella, Aerococcus, Anaerococcus, Prevotella and Veillonella [38]. Other urinary diseases such as chronic prostatitis/chronic pelvic pain syndrome exhibited in their urinary microbiome a higher bacterial diversity and enrichment of Clostridia class when compared to the control samples. The study also showed that these variations were also related to certain severity and clinical phenotypes as well as functional metabolism pathway perturbations [47]. In conclusion, all these studies demonstrate, in a certain way, that some urinary diseases are directly or indirectly associated with the urinary microbiome.

The Prostate Microbiome and Prostate Cancer

It has been shown that human microbiota can exert both direct effects on cancer development, as has been extensively studied in the context of infection-associated cancers, and indirect effects, such as regulation of the immune system, metabolism changes, and impacts on therapy [48]. For instance, *Helicobacter pylori* infection is a known cause of gastric cancer [49].

It is hypothesized that bacteria or viruses may influence various stages of prostate cancer from oncogenesis to disease progression. This may be via direct

interactions between the microbial environment and the prostate or indirect interactions such as immune modulation or alterations in metabolic pathways. Recent study demonstrated significant variations in microbial populations in prostatic secretions, voided urine and seminal fluid from patients diagnosed with prostate cancer or benign prostatic hyperplasia. It was shown that patients with prostate cancer had an increase in Bacteroidetes, Alphaproteobacteria, Firmicutes, Propionicimonas, Schingomonas and Ochrobactrum loads, they also showed higher levels of *E. coli* in seminal fluid and prostatic secretion and less in urine samples and high levels of Enterococcus in seminal fluid but not significant in prostatic secretion and in urine samples [50]. Therefore, the authors suggested a possible role for the microbiome in the pathobiology of prostate cancer.

Infections, Inflammation and Cancer Risk

Although microorganisms are commonly found within prostatic tissue no single microorganism has been unequivocally linked to prostate oncogenesis [51]. Interestingly, Sfanos et al. recently proposed that rather than a single microorganism causing prostate carcinogenesis, it is more likely that prostatic inflammation, which can be induced by multiple different organisms, is the more important causative factor [51].

Recent epidemiological studies have associated prostatitis with an increased cancer risk [52–54]. Although these studies are limited due to detection bias, an elegant prospective study by Platz et al. found a positive association between the presence of inflammation in benign tissue and subsequent development of prostate cancer [55]. Patients enrolled in the Prostate Cancer Prevention Trial with a negative end of study biopsy had their biopsy specimens assessed for inflammation; those with the presence of inflammation in benign tissue were associated with an increased risk of future development of prostate cancer.

In animal models, introduction of a bacterial stimulus to the prostate has been shown to elicit an inflammatory reaction which lasts for months after the original infection is eradicated [56, 57] . This chronic inflammation results in epithelial hyperplasia, oxidative DNA damage and down regulation of the tumour suppressor protein Nkx 3.1 in prostate epithelial cells [58, 59], all of which potentially leading to oncogenesis. Interestingly, inducing chronic inflammation by *E. coli* in a mouse model was shown to increase the rate of development of invasive prostatic adenocarcinoma [60].

Urinary Microbiome and Prostate Cancer

The discovery of a urinary microbiome has important pathophysiological implications in terms of prostatic disease including prostate cancer due to their anatomical proximity.

Shrestha et al. compared the urinary microbiome of men with biopsy proven prostate cancer to those with a benign diagnosis [36]. They noted a higher

concentration of pro-inflammatory bacteria such as *Streptococcus anginosus, Anaerococcus lactolyticus, Anaerococcus obesiensis, Varibaculum cambriense* and *Propionimicrobium lymphophilum* in a subset of cancer patients. These bacteria had previously been associated with either colorectal and head and neck cancers or urinary tract infections. The presence of those pro inflammatory bacteria may lead to chronic prostatic inflammation which may in turn lead to oncogenesis.

Gastrointestinal Microbiome and Prostate Cancer

Our gastrointestinal microbiome represents an important environmental factor to which we are constantly exposed. Mechanisms by which the GI microbiota may cause oncogenesis are multiple and include: Microbial dysbiosis and production of oncogenic metabolites or reduced production of tumour suppressive metabolites, immune modulation mediated by microorganism associated molecular patterns and production of genotoxins causing DNA damage.

As well, specific colonic bacteria may also be carcinogenic. A study by Golombos et al. [61] demonstrated a higher concentration of pro-inflammatory bacteria *Bacteroides baciliensis* in patients with prostate cancer while the benign controls had a higher concentration of *Firmicutes prausnitzii*. Interestingly, levels of *B. baciliensis* were also found to be present in a higher concentration in patients with colorectal adenoma and cancer [62]. *F. prausnitzii* produces butyrate from acetate. Butyrate is a short chain fatty acid which serves as an energy source for colonocytes. In addition, it also has anti-inflammatory properties [63]. In in vitro models, it can cause apoptosis, inhibits proliferation and promotes cellular differentiation [64].

The intestinal microbiome also provides a source of various bioactive substances such as folate, arginine, riboflavin, biotin and butyrate [65]. Folate is of particular interest with regards to prostate cancer. A secondary RCT analysis by Figueiredo et al. [66] showed that patients who received folate supplementation had a higher risk of developing prostate cancer whereas non supplement controls who had dietary folate intake and baseline plasma folate levels showed a trend for lower risk of prostate cancer. This may suggest that natural sources of folate are protective. A recent study by Liss et al. seemed to confirm those findings by demonstrating a higher prevalence of folate producing microbiota in patients without prostate cancer. That same study by Liss et al. [67] showed that bacteria associated with carbohydrate metabolism were in abundance while those responsible for B vitamin and Arginine production were lacking in patients with prostate cancer.

Some bacteria within the GI tract produce B glucoronidase which by its deconjugating activities increases the levels of circulating free estrogens. There is some evidence that higher levels of circulating estrogen may contribute to prostate cancer development by interacting with DNA and causing the development of apurinic sites, eventually leading to mutations responsible for prostate cancer [68].

Gastrointestinal Microbiome and Immunotherapy

There is growing evidence that prostate cancer is immunogenic. Sipuleucel-T which is composed of ex vivo activated autologous mononuclear cells is known to be effective in metastatic castrate resistant prostate cancer. Moreover, an RCT by Beer et al. showed improved progression free survival with use of an anti CTLA4 agent. There has also been some case reports of long term complete responses with Immune Checkpoint Inhibitors in the metastatic prostate cancer setting [69]. There is emerging evidence that therapeutic efficacy of some immunotherapeutic and chemotherapeutic agents is dependent on the gut microbiota [48, 70, 71]. In animal models, it has been demonstrated that the GI microbiome is essential to the therapeutic effect of cyclophosphamide, Anti-CTLA4 and Anti-PDL1 immunotherapies [48, 70, 71]. Eradicating commensal bacteria by way of antibiotic treatment resulted in elimination of the therapeutic efficacy of the above agents. Interestingly, increased efficacy of anti PDL1 therapy was achieved by feeding mouse models a specific strain of Bifidobacterium [71]. In another study, Vetizou et al. [48] showed that T-Cell responses specific to *Bacteroides fragilis* were essential for anti-CTLA4 agents to inhibit tumour growth in mouse models. Hence the gut microbiome influences the efficacy of immunotherapeutic agents and could potentially be modified to enhance tumour responses to these agents.

The Microbiome of the Prostate Tumour Microenvironment

Cavaretta et al. [72] analysed the tumour, peri-tumour and non tumour microbiome from patients having undergone a Radical Prostatectomy. This study confirmed that the prostate microenvironment is non sterile. Moreover, at each taxonomic level, a gradual change was noted in the concentration of some bacterial groups between the tumour, peri-tumour and non tumour regions of the prostate. Indeed, the microbiome of the peri-tumour region were more similar to the tumour region than the non tumour region. This could be due to the prostate tumour microenvironment promoting the growth of a specific microbial population. These in turn may influence tumour progression by modulating host immune response and extracellular matrix composition.

Propionibacterium spp. were found in higher abundance in the tumour/peri - tumour samples which is consistent with their pro inflammatory role and supports their association with prostate cancer [56, 73]. Corynebacterium spp. was also relatively more abundant in tumour/peri-tumour regions, possibly related to the capacity of these species to form biofilm and bind to fibronectin present in the extracellular matrix. On the other hand, a higher concentration of streptococcus spp. was found in non tumour areas hence supporting its role as a resident of the normal prostate microbiome. In summary, the gastrointestinal, urinary and prostate tumour microenvironment may play an important role in prostate carcinogenesis and even response to therapy. Further research will help to define possible microbial biomarkers for

prevention, early diagnosis and risk stratification of prostate cancer as well as possible targets to attempt modulation of the process of carcinogenesis and for therapy.

Conclusions

The study of the microbiome of the prostate reveals a complex role of the microbes and prostate health, function, inflammation and even carcinogenesis. Further research in the relationships between the microbial environment and the prostate will need to be explored so that new biomarkers and treatments for both prostatitis and prostate cancer are developed.

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Beyond Bacteria: The Mycobiome and Virome in Urology

13

Louisa Ho, Simran Parmar, and Kymora B. Scotland

Abbreviations

AdV	Adenovirus
BKV	BK virus
CMV	Cytomegalovirus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
JCV	JC virus
NAAT	Nucleic acid amplification tests
NGS	Next-generation DNA sequencing
PML	Progressive multifocal leukoencephalopathy
RSV	Respiratory syncytial virus
SOT	Solid organ transplant
UTI	Urinary tract infection
VLP	Virus-like particles
VZV	Varicella zoster virus

L. Ho · S. Parmar
The Stone Centre at Vancouver General Hospital, Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada

K. B. Scotland (✉)
University of British Columbia, The Stone Centre at Vancouver General Hospital, Jack Bell Research Centre, Vancouver, BC, Canada
e-mail: kymora.scotland@ubc.ca

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Introduction

The microbiome associated with the healthy urinary tract can change in urologic disorders. Lower urinary tract infections (UTIs) are common, and are most often caused by bacterial pathogens, and also occasionally by fungi or viruses. The bacterial microbiome of the urinary tract has begun to be better characterized, but much less is known about the viral and fungal microbiota. Overall community burden, composition, and virulence states likely interact and respond to host health status, and minor perturbations of these microbial communities may potentially bias the host from symbiosis towards disease. There is much interest in identifying new diagnostic, prognostic, and predictive microbiome-based biomarkers that could be used in clinical urology practice to modulate the microbiome to improve urinary tract health [1]. In this chapter, we discuss the viral and fungal microbiota.

Human Virome

The microbiome pays a role in modifying fundamental human physiology. Innovations in genomic sequencing coupled with the development of high resolution microscopy techniques have led to the discovery of the human virome (Fig. 13.1). However, the continued investigation into the virome is in its infancy with more questions than answers about newly discovered viral sequences [2]. Study of the virome has been made difficult by the lack of a shared genetic marker among viruses as is the case with bacteria and their 16S ribosomal RNA gene. This means that characterization of the virome cannot depend on the much less

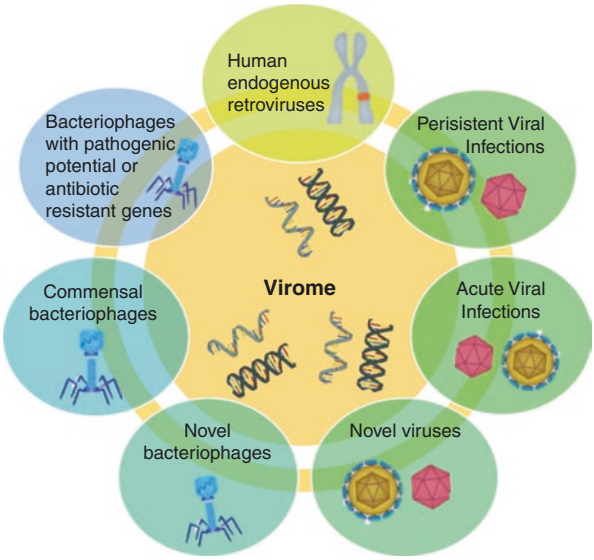

Fig. 13.1 Human Virome, also referred to as the viral metagenome, is the collection of nucleic acids, both RNA and DNA, that make up the viral community found in the human body. The circles represent components of the virome based on metagenomic sequencing. The components are not mutually exclusive

Table 13.1 Common viruses isolated from human urine [8]

BKV	Polyomavirus	Gammatorquevirus
Adenovirus	BK	Begomovirus
Mardivirus	Polyomavirus	Ilarvirus
Cytomegalovirus	JC	Sapovirus
Alpha Papillomavirus	Totivirus	Enterovirus
Beta Papillomavirus	Lentivirus	Mastadenovirus
Gamma Papillomavirus	Alphatorquevirus	Unclassified Papillomaviridae
Chlorovirus	Betatorquevirus	Unclassified Anelloviridae

expensive ribosomal sequencing. Instead, viral identification must currently rely on the identification of markers associated with different taxonomic groups and on the degree to which sequences are similar to those from known viruses [3].

Urinary Virome

The urinary tract has a robust community of viruses that are present in healthy, asymptomatic, immunocompetent individuals. These viruses generally are only pathogenic in an immunocompromised host. Virus-like particle (VLP) concentrations of up to 10^7 VLP have been reported, but of the total analyzed human and viral proteins in urine, viral proteins only constitute <0.2% of the total identified proteins. This highlights the very low abundance of viral proteins in human urine, with no reported patterns of variation in the membership of viral communities that were attributable to bacterial infection status.

Common urinary virobiota include BK-virus (BKV) [4], adenovirus (AdV) [5, 6] and human papillomavirus (HPV) [7]. None of the identified HPV genotypes were ones associated with cervical and rectal cancers. Of note, some common viruses with clinical significance, such as Epstein-Barr virus (EBV) and Herpes simplex virus (HSV), were not detected in the urine, most likely because they are localized in other body compartments (Table 13.1). Many of the viruses that have been isolated in the urine are bacteriophages. In addition, viruses used as pesticides/insecticides (*E. obliqua* nucleopolyhedrovirus, *Euproctis pseudoconspersa* nucleopolyhedrovirus, *Helicoverpa armigera* nucleopolyhedrovirus, *H. zea* single nucleopolyhedrovirus) have also been detected in human urine.

Viral UTIs

Epidemiology

Risk of viral UTI is associated with immunocompromised states due to immune suppression or abnormal immune function. Viral hemorrhagic cystitis is most commonly

seen after bone marrow transplantation, but is also seen in the renal transplant population. Other conditions associated with viral UTI include other solid organ transplantation, hematopoietic malignancies, chemotherapy, HIV infection, congenital immunodeficiency, pregnancy, diabetes, alcoholism, malnutrition and liver cirrhosis [9].

Signs and Symptoms

A viral UTI is defined as the presence of an identifiable viral organism with inflammatory symptoms. Symptoms include frequency, urgency, hematuria, genital or lower abdominal pain and pyuria as well as hematospermia in males. The most common presenting symptom of a viral UTI is hemorrhagic cystitis [7].

Diagnosis

Diagnosis of a viral UTI is more challenging than a bacterial UTI. Viruses are too small to be detected by direct light microscopy, although the presence of certain viruses, such as CMV and BKV, may be suspected by characteristic changes on urine cytology. Viral cultures are possible for AdV, CMV, HSV, enteroviruses, influenza, mumps, parainfluenza, RSV, and VZV, but may take up to 14–28 days to speciate [7]. Viral culture is not applicable for detection of Coxsackie A viruses, hepatitis viruses, arbovirus, parvovirus, HPV, reovirus, measles virus and gastrointestinal viruses.

Thus, diagnosis of a viral UTI is based on molecular techniques. Viral testing in the clinical laboratory typically relies on single-target nucleic acid amplification tests (NAATs) with real-time quantitative polymerase chain reaction of urine or blood samples. It is the preferred method for clinical diagnosis as it allows for the quantification of viral load. However, PCR assays are restricted to a limited number of viruses per assay, and are limited in identification of virus subtypes [3].

In the research setting, genomic approaches have been used to comprehensively and quantitatively describe the urinary virome. Virus microarrays offer breadth in virus detection, but have suboptimal sensitivity. Next-generation DNA sequencing (NGS) technology with high-throughput shotgun metagenome sequencing can identify nearly complete virus genomes, including genetic polymorphisms and virus subtypes, with high accuracy and throughput directly from clinical samples. It has led to the discovery of emerging viruses, previously unrecognized classes of indigenous human viruses, and virus variants. However, the clinical application of NGS is limited, as the clinical sensitivity currently remains lower than those for virus-specific NAATs, and quantitative viral testing via NGS has not been extensively studied [10, 11].

Special Considerations: Transplant

In solid organ transplantation (SOT), immunosuppression that is used to prevent and treat organ rejection results in a heightened and prolonged risk for opportunistic

viral infections. The development of de novo viral infections or reactivation of latent viral infections in transplant patients causes significant morbidity and mortality.

Primary infection with BKV and JC virus (JCV), both members of the *Polyomaviridae* family, occurs in healthy individuals, often during childhood, and is detected by sero-positivity of anti-viral antibodies. Following infection, these viruses commonly become latent; however, they persist in the urinary tract and can reactivate and become dominant in the context of immunosuppression following transplantation. One study found that the composition of the virome was different in stable grafts versus acute rejection, BK viral nephritis and chronic allograph nephropathy [8]. BKV has been linked to interstitial nephritis, ureteral stenosis, and hemorrhagic cystitis. JCV has been associated with renal tubular epithelial infection and progressive multifocal leukoencephalopathy (PML). BKV, CMV, and AdV are the most common viral pathogens isolated in hemorrhagic cystitis after renal transplantation. Not only are transplant recipients at risk for multiple opportunistic viral infections, but the subtypes appear to differ as well, with potential for increased pathogenicity [12]. Transplant-specific multiplexed syndromic panels are not currently available and a large number of potentially disease-causing viruses likely remain uncharacterized.

Treatment

Cidofovir is a drug of choice in viral UTIs because it is active against the most common viral pathogens. Adenoviral hemorrhagic cystitis is usually self-limiting, and treatment depends on the clinical picture [10].

Clinical Implications

To date, studies investigating virome diversity have not shown clear associations with differences in the overall health of the urinary tract. While more data are still needed, recent detection of HPV in the human urinary virome using metagenomics could represent a non-invasive method to screen for HPV in patients [7].

Pesticides are widely used in agriculture and home garden use, and in addition to the toxic effects of acute exposures, epidemiologic studies suggest associations of low dose chronic exposures with adverse developmental effects, particularly in children [13]. The finding of viral proteins associated with pesticides in human urine samples has public health implications. It may also represent a method of monitoring environmental exposure to these compounds [9].

Mycobiome

As a result of the observation that immunosuppressed patients given antibiotics may develop candidiasis, we have long suspected that fungi inhabit the human body. However, results of the first attempt at description of the mycobiome were only

recently presented [14]. The mycobiome is thought to be as low as 0.1% of the total microbiome [15]. Because of this low abundance, isolation of fungi relies on selective culture methods as well as next-generation sequencing [16].

Ongoing characterization of the mycobiome has revealed diverse fungal communities throughout the body, suggesting that commensal fungi may play critical roles in human health. One example of this is the vaginal mycobiome which has only recently begun to be studied. A report by Drell and colleagues revealed that the fungal constituents of the vagina were composed of 57% Ascomycota species including *Candida*. This work also identified the Ascomycota *Davidiellaceae*, *Cladosporium*, *Eurotium*, *Alternaria*, and the basidomycota *Rhodotorula* as minor constituents [17]. Fungal dysbiosis has been implicated in vaginal yeast infections among other diseases. Continued study may give insight into better management of this and other diseases.

Urinary Mycobiome

The human urinary tract can also be host to a diverse population of fungi, but the fungal microbiota community profiling data are currently limited. In the lower urinary tract, *Candida* species, belonging to the Saccharomycetes class, are normal commensals in humans, and the predominant *Candida* species present is *Candida albicans*, followed by *Candida glabrata* and then *Candida tropicalis* [18]. In addition, there has been a reported increase in the incidence of candiduria caused by more resistant non-*Candida albicans* species. The Saccharomycetes class had been the only fungal class documented in urine, however, one recent study using NGS rather than conventional culture-based characterization to profile urinary fungi from a range of asymptomatic patients detected a much wider breadth of fungal taxa not identifiable using current available fungal sequence databases, highlighting the nascent status of this field [19].

In the upper urinary tract, Candida can cause renal candidiasis, while invasive fungal species (*Cryptococcus neoformans*, *Aspergillus* sp., *Mucoraceae* sp., *Histoplasma capsulatum*, *Blastomyces* sp., *Coccidioides immitis*) may infect the kidneys as part of a systemic or mycotic infection.

Fungal UTIs

Epidemiology

Candiduria, the presence of *Candida* species in urine, is a common clinical finding, particularly in hospitalized patients. Within the hospital setting, candiduria is especially common in intensive care units (ICUs). The use of increasingly aggressive treatments has prolonged the lives of patients susceptible to candiduria, namely the immunosuppressed. Long-term urinary catheterization is considered to be the most significant risk factor for candiduria followed by antibiotic use and diabetes.

Table 13.2 Risk factors for Candiduria and Candida UTI [18, 19]

Diabetes mellitus	Congenital abnormalities of urinary tract
Extremes of age	Structural abnormalities of urinary tract
Female sex	Broad-spectrum antibiotics
Prolonged hospitalization	Bladder dysfunction
ICU admission	Urinary stasis
Renal transplantation	Bladder stones
Instrumentation of urinary tract	Indwelling urinary tract devices
Concomitant bacteriuria	

Nosocomial candiduria is associated with high morbidity, mortality, and long hospitalization, involving high costs for the healthcare system. In one study, the incidence of candiduria in the elderly population was 10.3% [20]. Renal transplantation increases the risk because of the combination of indwelling catheters, stents, antibiotics, anastomotic leaks, obstruction, and immunosuppressive therapy [18]. Table 13.2 indicates common risk factors for candiduria.

Renal candidiasis is usually spread hematogenously from systemic candidiasis and commonly originates from the GI tract. Ascending pyelonephritis is uncommon but may be seen in patients with diabetes, renal insufficiency, obstructive uropathy, or patients with nephrostomy tubes or ureteric stents that have been colonized [21]. Candidemia rarely results from asymptomatic candiduria in the absence of obstruction or urinary tract instrumentation [22].

Signs and Symptoms

A fungal lower UTI is defined as the presence of an identifiable fungal organism with inflammatory symptoms. Most patients with candiduria are asymptomatic. Cystitis due to fungal infection may result in frequency, urgency, hematuria, genital or lower abdominal pain, pyuria, or hematospermia. In patients with poorly controlled diabetes, a presenting symptom may be pneumaturia due to emphysematous cystitis [18]. Fungus balls or bezoars may cause symptoms of urethral obstruction [21].

Patients with renal candidiasis may have antibiotic-resistant fever, candiduria, and unexplained deteriorating renal function. Fungus ball elements in the ureter and renal pelvis frequently cause hematuria and urinary obstruction. Occasionally, papillary necrosis or intra-renal or perinephric abscesses cause pain, fever, hypertension and hematuria. Patients may also have manifestations of candidiasis in other sites (e.g., CNS, skin, eyes, liver, spleen).

Complications of *Candida* infection include emphysematous cystitis or pyelonephritis and fungus balls in the renal pelvis, ureter, or bladder. Bezoars may form in the bladder. Lower or upper urinary tract obstruction may occur. Papillary necrosis and intrarenal and perinephric abscesses may form. Although renal function often declines, severe renal failure is rare without post-renal obstruction [20].

Diagnosis

Diagnosis of fungal UTI is made with urine culture and evidence of cystitis or pyelonephritis. Diagnosis of fungal UTI is complicated by the fact that *Candida* species are a known commensal of the genitourinary tract. *Candida* colonization differs from infection in that infection produces tissue reaction, and the level at which candiduria reflects true *Candida* UTI and not merely colonization or contamination is unknown. In addition, urinary samples may not necessarily reflect the bladder microbiota, as it has been hypothesized that fungi may attach directly to the urothelium to form mixed biofilms, or remain quiescent within cells of the bladder wall [18, 19].

Special Considerations: Transplant

While the most common pathogens are bacterial, fungal UTI may cause serious complications that influence graft success and patient survival. Fungal infection rates have been quoted from 3% [23] to 8.5% [24].

Treatment

Asymptomatic candiduria rarely requires therapy. Fungal colonization of catheters does not require treatment. Candiduria should only be treated in symptomatic or high-risk patients (neutropenic patients, renal transplant patients, patients undergoing urologic manipulation). Foley catheters and urinary stents should be removed if possible [18, 25].

For symptomatic cystitis, fluconazole is the main drug used for its efficacy and low rate of complications (see Table 13.3). Other options include the addition of amphotericin B for resistant species [26]. The addition of flucytosine can help eradicate candiduria due to non-*albicans* species of *Candida*; however, resistance may emerge rapidly when this compound is used alone. Bladder irrigation with amphotericin B may transiently clear candiduria but is no longer indicated for cystitis or pyelonephritis. Since azoles other than fluconazole and all echinocandins are poorly excreted in urine they have been found to be less effective in candiduric patients [27]. Even with apparently successful local or systemic antifungal therapy for candiduria, relapse is frequent, and this likelihood is increased by continued use of a urinary catheter.

Table 13.3 Treatment regimens for Candidal infections [22]

	Fluconazole sensitive	Fluconazole resistant
Cystitis	Fluconazole 200 mg po daily × 14 day	Amphotercin B 0.3–0.6 mg/kg IV daily × 14 day
Pyelonephritis	Fluconazole 400 mg po daily × 14 day	Amphotercin B 0.5–0.7 mg/kg IV daily × 14 day
		+/− Flucytosine 25 mg/kg po four times daily

Clinical Implications

Candiduria may be the only indicator of a more serious invasive candidiasis, especially in immunocompromised patients. With regards to other mycobiota of the urinary tract, profiling data are still limited, and our understanding of how the fungal mycobiota contributes to the regulation of urinary tract health, function and inflammation is unclear. This is a nascent field, and further research is needed to better characterize the role that fungi play in urologic health and disease.

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Metagenomics and the Microbiome

14

Victor Wong and Dirk Lange

What Is Metagenomics?

Genomics is the interdisciplinary field of science focusing on structure, function, evolution, mapping and editing of genes. Historically, microbial genomics involved the isolation of microbes for genetic analysis through culturing methods to identify the genetic profile of species within a given sample. In recent years, metagenomics has risen as a new frontier both as a research field and approach to circumvent the unculturability and genomic diversity of most microbes, the biggest roadblocks to advances in clinical and environmental microbiology [1]. The discipline of metagenomics represents the study of population genomics at the level of microorganisms, referencing the notion that a collection of genes from a given environment can be analyzed in a way analogous to that of a single genome, offering a powerful lens into the microbial world that has the potential to revolutionize the clinical sciences [2].

History of Metagenomics

Conventional genomics began with the culturing of identifiable cells as a DNA source for analysis. However, early researchers investigating the preliminary notions of metagenomics hypothesized that the major weakness of conventional genomics

V. Wong
The Stone Centre at Vancouver General Hospital, Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada

D. Lange (✉)
University of British Columbia, The Stone Centre at Vancouver General Hospital, Jack Bell Research Centre, Vancouver, BC, Canada
e-mail: dirk.lange@ubc.ca

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is that groups of microorganisms cannot be analyzed if they cannot be cultured, and thus cannot be sequenced for genomic analysis. To overcome this hurdle in the field of genomics, the bacteria-specific 16S rRNA sequence was the main focus of early metagenomics as this sequence was relatively short, often conserved within a species and generally different between bacterial species [3]. This led to the first report of isolating and cloning of bulk DNA from an environmental sample, published by Pace and colleagues in 1991 [4].

The Metagenomic Analysis Strategy

One of the strongest potentials of metagenomics when compared to conventional genomics lies in the ability to detect trends and correlations indicative of interactions between the microbial world and the environment. In present day, metagenomic analyses are affordable and accessible to the average microbiology project, allowing for the generation of massive sequence outputs [5]. Figure 14.1 outlines the workflow for metagenomics analysis. The first step of a metagenomic analysis after acquiring of a sample involves the sequencing of DNA. Currently, two main approaches are utilized to generate the bulk metagenomic data: Shotgun metagenomics and high-throughput sequencing. Shotgun metagenomics involves the random sheering of DNA following its extraction, resulting in the formation of many short sequences which are then reconstructed into a consensus sequence. Shotgun metagenomics provides information about which organisms are present and what metabolic processes are possible in the community [6]. High-throughput sequencing (HTS) allows for the sequencing of multiple DNA molecules in parallel, enabling hundreds of millions of DNA molecules to be sequenced at a time. This advantage allows HTS to be used to create large data sets, generating more comprehensive insights into the cellular genomic and transcriptomic signatures of various diseases and developmental stages [7]. These sequencing techniques used in metagenomics bypasses the cloning and culturing requirements of traditional genomic studies before sequencing can be conducted, removing one of the main biases and bottlenecks in microbial environmental sampling.

Consequently, the data that are generated by metagenomic experiments are both enormous and inherently noisy, containing fragmented data representing as many as 10,000 species of microbes [8]. Bioinformatics is used to acquire relevant biological information from the generated following sequencing so that the metagenomic dataset generated at the end of the experiment can be analysed. Contaminating eukaryotic genomic DNA sequences and other genomic data not relevant to the study are removed, allowing for the assembly of DNA sequences identifying microbes and their relevant abundance, genes, and gene functions. Coding regions of the genes of interest in assembled contigs are annotated based on homology or by known intrinsic features of sequences from suspected related organisms. Once gene annotation has occurred, genomic binning is conducted to measure species diversity within the produced metagenomic dataset [9]. Genomic binning can be conducted in two ways. Similarity-based binning is used to rapidly search for phylogenetic markers

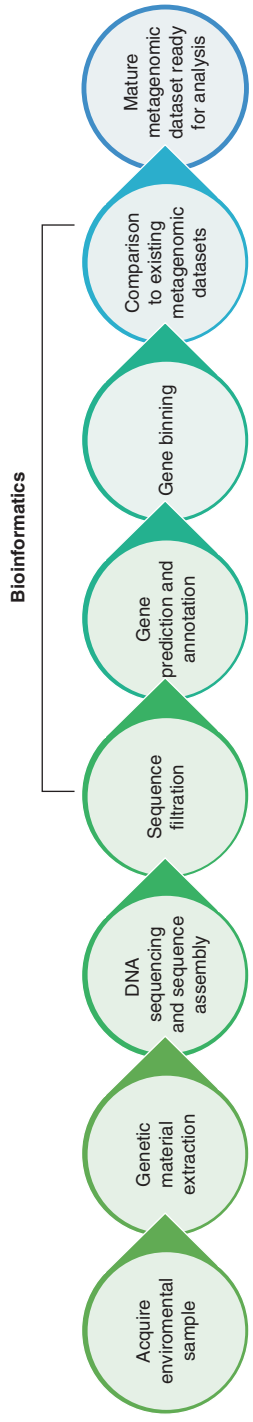

Fig. 14.1 Metagenomic analysis workflow

or otherwise similar sequences in existing public databases and composition-based binning using intrinsic features of the sequence [8]. Once binning has occurred, the metagenomic dataset is compared with existing or known datasets through the use of programs such as MEGAN (MEta Genomic ANalyzer) to explore taxonomic diversification of the dataset, comparing the resulting sequences with gene sequences from GenBank in NCBI [10]. Gene annotations provide the "what", while measurements of species diversity provide the "who" [11]. Metagenomic datasets derived from a given community (e.g. patients sharing with similar urologic diseases), can identify microbial groups which are responsible for conferring specific characteristics of given environment and is the main goal of metagenomic studies in the clinical sciences, providing additional insight into the function of complex microbial communities and their role in host health [12].

Metagenomics in Medicine

Clinical Relevance of Metagenomics

Currently diagnosis of a vast majority of microbial diseases is carried out using traditional culture-based methods. In a clinical context, culture-based methods can fail to isolate all disease-causing organisms [13] especially in a multi-species scenario and are time consuming and labor intensive [14]. Although metagenomics analysis is not yet standard clinical practice, utilizing a metagenomic approach clinically has the potential to identify and characterize specific bacterial and viral pathogens causing a given condition [15]. Data generated can be utilized as functional information to understand complex infections at a genetic level as well as how members of the microbiome contribute to disease through interactions with host physiology [15].

Three themes have emerged from the application of microbial ecology to clinical microbiology, where metagenomics can have its applications. Within the clinical laboratory, most bacteria cannot be isolated in pure culture, so molecular methods will report a wider range of organisms than culture and are generally more sensitive [16]. Differences in host-associated microbial communities can influence the balance between health and disease in conditions not normally thought of as microbial or infectious in origin (e.g. inflammatory bowel disease, cancer or obesity) [17]. Lastly, developments in infection research have suggested that interactions between organisms in a community can influence disease outcomes and in some cases it might even be appropriate to treat a whole microbial community as a pathogenic entity, as opposed to the notion that a single pathogen causes a single disease [19].

The Potential of Clinical Metagenomics Application

Prior to a discussion of how metagenomics can be used for diagnostic purposes in the clinical setting, it is worth reviewing the problems associated with existing

diagnostic approaches. Microbial diagnostic techniques currently used in the clinical setting today were developed over a century ago (the detection and characterization of bacteria through microscopy and gram staining, and culturing of clinical isolates) [20, 21]. In situations where microscopy is cumbersome, unrewarding or difficulties in culturing is encountered, culture-independent approaches to pathogen detection are used, including immunoassays and detection of nucleic acid sequences [22]. Although practical, these approaches are generally target-specific and thus lack the ability to detect unsuspected pathogens, resulting in a battery of tests that may have to be applied to each sample, each requiring optimization and standardization [21].

Clinical diagnostic metagenomics brings the promise of an open-ended, assumption-free one-size-fits-all workflow that could be applied to any specimen to detect any kind of pathogen [21]. Given the rapid development of tools targeted for pathogen identification, and likely future improvements in the ease, throughput and cost-effectiveness of sequencing, twinned with commoditization of laboratory and informatics workflows, one can foresee a tipping point when a unified automated metagenomics-based workflow will start to compete with the plethora of methods currently in use in the diagnostic laboratory, while also delivering additional useful information (e.g. genomic epidemiology, antimicrobial resistance, virulence). Given the rapid development of tools targeted for pathogen identification, it is feasible that metagenomics will play a key role in the clinical laboratory in the near future.

A Future for Clinical Diagnostic Metagenomics

Given its significant potential at advancing clinical diagnosis, the question that remains is what stands in the way of its application in the field? Diagnostic metagenomics is still currently in its infancy. A study in 2013 using metagenomics to investigate diarrhea samples that were positive for Shiga-Toxigenic *E. coli* showed a sensitivity of only 67% compared to culture [15]. One of the foremost problems with diagnostic metagenomics is the handling, interpreting, and making use of the tremendous amounts of data obtained [23]. Metagenomics to be used in the clinical setting not only requires the knowledge of the Manual of Clinical Microbiology but also all of environmental microbiology, International Journal of Systematic and Evolutionary Microbiology and the entirety of the NCBI taxonomy database [23]. This will require the formation of a not currently existent workforce specialized in the bioinformatic analysis of metagenomic data to translate the sequences obtained into results that are clinically applicable. Another major challenge is the cost of a single diagnosis. One of the main benefits of the current clinical microbiology testing paradigm is that it is cost-effective [23]. For comparison, current clinical microbiological markups in the clinical microbiology lab convert <$5 of reagents and minutes of technician time into $200–500 reimbursements, compared to current metagenomic testing prices in the low four figures ($2000–3000) per sample [23]. Hence, for diagnostic metagenomics to be implemented widely, the costs associated with such testing will need to be reduced.

That said, when the implementation of clinical diagnostic metagenomics does occur, it will become possible to serially characterize human microbiomes to investigate for disease associations. This could lead to personalized medicine guiding the use of narrow-spectrum antibiotics to reduce disruption of microbiomes or identifying specific probiotics to restore an individual's microbiome to a healthy state [24]. Although the widespread implementation of diagnostic metagenomics has not occurred yet, this has not stopped researchers from applying such techniques at a research level. As examples, metagenomics has been used to diagnose *C. jejuni* infections from fecal samples [13], *E.coli* from urine samples [25], and lymphocytic choriomeningitis virus in fatal infections in transplant recipients [26]. These studies clearly show the significant potential for metagenomics in disease diagnosis.

Given the significant role for metagenomics in microbiome analysis of various organ systems, it can serve as an important tool to track the success of treatments aimed to increase the abundance of bacterial populations associated with positive health outcomes. That said, a link between microbiome composition and specific disease states will need to be established prior to the development of bacterial-based treatment options. Such techniques have been applied in fecal microbiota transplantations, where shotgun metagenomic techniques were used to track the colonization of donor microbial communities over time from the initial transplantation [27]. Colonization trends tracked with assembled metagenomic profiles at varying time points were compared to the Human Microbiome Project (HMP), where the metagenomic profiles of successful cases mirrored that of the HMP, while failures resulted in missing profiles [27]. This study suggests that large-scale metagenomic surveys can be useful in the prediction of successful treatments associated with microbiome abnormalities in the future. Analogies from this study can be drawn and applied in various diseases associated with microbiomes, the composition of which can be tracked over time via changes to metagenomic profiles as the treatment progresses.

Metagenomics in Urology

Microbiome of the Urogenital Tract

Given the emerging evidence of the microbiota's role in maintaining urinary health metagenomics is becoming an important tool in urologic research. Studies of the urinary microbiota have identified differences between healthy populations and those with urologic diseases [28]. Microorganisms at sites distal to the kidney, bladder and urethra are likely to have a profound effect on urologic health, both positive and negative, owing to their metabolic output and other contributions [28]. Connections between the gut microbiota and renal stone formation have already been discovered [28].

While links are being made, the specific relationship between these actively metabolizing organisms and urogenital health has yet to be completely elucidated. Given the role of the kidneys and bladder in filtration and storage of waste, respectively, microbial profiles at these sites may influence important absorptive

functions, and alterations might affect urinary homeostasis. Whether the microbiomes of these sites are predictive of the risk of urological disease remains to be elucidated [28]. Given the potential importance of the urinary microbiota to an individual's health and disease manifestation, metagenomic research of the urinary and gut microbiomes is warranted and will lead to important findings on how microbiomes associated with the urinary tract influence the overall health of the host.

A Role of Metagenomics in Urology

Conventional microbiological methods are inadequate to fully determine the diversity of bacteria present in urine [29]. To understand the role for metagenomic investigations in urology, we must first investigate the current clinically available urological tests. Urinary tract infections are among the most common bacterial infections [30], and are usually classified as uncomplicated and complicated, but more recently also by risk factors and severity grading depending on the clinical presentation [31]. The diagnosis requires clinical symptoms and evidence of living bacteria in the urine, usually quantified by numbers of colony forming units per milliliter (CFU/ml). Culture tests and urine microscopy have been the gold standard for diagnosing UTI [31, 32]. However, no fixed bacterial count has been considered conclusive for significant bacteriuria in UTIs [33]. The currently used urine culture method for diagnosis is limited to detecting easily culturable aerobic bacteria only and not fastidious and anaerobic bacteria [34]. The underlying idea has always been that urine from healthy subjects is sterile and a negative or positive urine culture has usually been taken as discriminative for an infection to be absent or present, respectively [34].

Metagenomics approaches appear to more comprehensively and quantitatively describe the urinary microbiome [35]. Recent metagenomic studies have identified a broad range of bacteria including non-culturable species in "sterile" bladder urine in healthy individuals [35]. Thus, sterile urine may be a myth and recent metagenomic findings on the urine microbiome encourage a discussion to redefine the criteria for urinary tract infections and non-infectious urological disorders with similar symptoms [35]. Diagnostic metagenomics used clinically in the future may enable clinicians to detect a wide range of bacteria in the urine of healthy individuals and patients with different urological disorders, better understand the underlying mechanisms and help guide individualized treatment [35].

Metagenomic analysis may prove to be a key factor in determining the critical microbiome community capable of preventing urolithiasis. Urolithiasis (Kidney Stones) affect up to 10% of people and can lead to pain, surgery, hospitalization and loss of time at work. Compositional analysis of kidney stones has revealed that over 80% of stones consist of calcium and oxalate derived from dietary and bodily processes. Individuals who have high levels of oxalate in their urine have a greater tendency to generate stones. Some patients, despite reducing their oxalate intake, still have high amounts in the urine [36–40]. The gut microbiome has been referred to as a metabolic organ that communicates with and complements our own human metabolic apparatus and has been associated with diabetes, obesity, cardiovascular

disease and urologic diseases [36]. Evidence has shown that urolithiasis patients may have distinct gut microbial profiles when compared to control subjects [36]. A diagnostic metagenomic application could be a plausible method of approach in analysing patient microbiomes to determine whether the presence or absence of gut microbiome members may result in urolithiasis [10]. Further treatment plans can be envisioned from this, drawing inspiration from gut microbiome modulations (e.g. fecal transplantation). With previous studies in the transfer of gut microbiome from diabetic-free patients into diabetic patients and improvements in insulin sensitivity as a result [36], gut microbiome manipulation may represent a novel preventative treatment for urolithiasis patients in a similar fashion.

Bacterial prostatitis is a bacterial infection of the prostate gland occurring in both young and older men. There are four classifications for prostatitis: acute, bacterial, chronic bacterial, chronic non-bacterial prostatitis/chronic pelvic pain syndrome, and asymptomatic inflammatory prostatitis. Only 8–16% of clinical cases of prostatitis can be attributed to bacterial origins, which can be diagnosed via positive urine and/or prostate secretion cultures [41]. Although relatively uncommon when compared to other causes of prostatitis, men who have one episode of bacterial prostatitis are more likely to have subsequent episodes and progress to chronic bacterial and nonbacterial prostatitis [41]. Expressed prostate secretions during typical urologic examinations make for convenient samples for metagenomic studies of the bacterial ecology in the male urogenital tract [42]. However, metagenomic studies on prostate secretions are still in their infancy [42]. Preliminary studies into the microbiome differences between patients with and without prostatitis has shed light on the spectrum of microorganisms typically found in expressed prostatic samples [42]. As more sequencing studies are being conducted on prostatic secretions, we are beginning to learn more about the potential presence of a prostate microbiome that may have an important role in health and disease. Once a link between the two has been established, expressed prostate secretions represent an easily obtainable sample that can be analyzed with metagenomics for the clinical diagnosis of prostatitis, and such techniques may be useful in the study of the bacterial ecology related to prostatitis.

Neurogenic bladder dysfunction refers to any condition that impairs the bladder and bladder outlet afferent and efferent signaling. This results in difficulty or inability to pass urine without catheterization. Metagenomic analysis of urine samples from neuropathic bladder cases have shown greater proportions of *Lactobacillus crispatus* in females and *Staphylococcus haemolyticus* in males [43]. The *Lactobacillus* community differed significantly among females dependent on bladder function. Irrespective of gender, subjects with neuropathic bladder had greater proportions of *Enterococcus faecalis, Proteus mirabilis and Klebsiella pneumonia* [43]. These metagenomic findings are clinically relevant to the neurogenic bladder patient population who face a disproportionately high risk of genitourinary complications [43]. Furthermore, neurogenic bladder metagenomic studies has shed light on the role of the genus *Actinobaculum* in neurogenic bladder dysfunction which were not previously detected by classic bacterial cultivation methods. The presence of this genus has been shown to strongly be associated with pyuria [43]. Moreover,

it is speculated that the presence of *Actinobaculum sp* in spinal cord injury induced neurogenic bladder dysfunction may influence abnormal urinary findings and standard urine culture data and may lead to misinformed antibiotic therapies [43].

Urgency urinary incontinence is a poorly understood urinary condition characterized by symptoms that overlap urinary infection, including urinary urgency and increased frequency with urinary incontinence [44]. Given the significant overlap of incontinence symptoms with those of urinary tract infections, it is possible that incontinence may have a microbial component [44]. Using metagenomic analysis of urine samples comparing women with and without incontinence, significant difference between the frequency and abundance of bacteria has been shown [44]. Within the incontinence population, *Lactobacillus* and *Gardnerella* were the two most frequently detected genera [44]. Urine collected from female patients with urgency urinary incontinence was more likely to contain *Actinomyces*, *Aerococcus*, and *Gardnerella* and less likely to contain *Lactobacillus* than urine collected from female patients without urgency urinary incontinence [44]. Furthermore, studies have indicated that females with more severe urgency urinary incontinence symptoms have decreased microbial diversity in their urinary microbiomes [45]. As such, differing urinary microbiomes characterized with metagenomic tools may result in the ability to clinically characterize the patients with urgency urinary incontinence for whom the diversity, presence, or absence of certain bacteria may play a key role in the pathophysiology of the disease.

In the future, the systematic and prospective use of metagenomic tools for disease diagnosis in urologic medicine may shed light on the role of unknown and unconventional microorganisms in the urinary tract or gut that may have clinical relevance towards urologic disease manifestation. Bacterial populations associated with healthy urologic states can also be derived from metagenomic studies and guide the prospective treatments of at-risk patients. This warrants further research on the potential applications of metagenomics in urologic sciences and may subsequently result in better diagnostic and treatment practices for urologic diseases in the future.

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Urologic Devices: Infection and Encrustation

15

Colin Lundeen and Kymora B. Scotland

Introduction

Historically, the urinary tract was considered sterile and infection was presumed to be related solely to the ascending movement of bacteria through the urethra. In recent years this theory has been disproven through the utilization of advanced bacterial detection methods. The concept of a microbiome, common in other organ systems, is increasingly being explored in relation to the urinary tract. As the body of knowledge on the urinary microbiome expands, questions have arisen surrounding its role in the infection and encrustation of urologic devices.

Background

Urologic implements including catheters and stents have been used for years to aid in drainage of the urinary tract. Unfortunately, device use in the urinary tract is associated with high rates of infection despite much research into specialized materials and coatings. Since the introduction of the Foley catheter in 1920, bacteriuria

C. Lundeen
The Stone Centre at Vancouver General Hospital, Department of Urologic Sciences, University of British Columbia, Vancouver, BC, Canada

K. B. Scotland (✉)
University of British Columbia, The Stone Centre at Vancouver General Hospital, Jack Bell Research Centre, Vancouver, BC, Canada
e-mail: kymora.scotland@ubc.ca

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associated with catheter use has been well documented [1]. Similar findings have been noted with ureteral stents [2, 3]. Colonization of devices can occur within days to weeks [4] and is often followed by bacteriuria which is nearly universal in patients with chronic indwelling catheters [5].

Colonization does not imply bacteriuria though, as many urine cultures will be negative despite a positive device culture [2]. Even less common than asymptomatic bacteriuria is the occurrence of symptomatic urinary tract infections [6]. Nonetheless, due to the prevalence of urinary tract device use it is estimated that the urinary tract accounts for up to 40% of nosocomial infections. Approximately 80% of these are related to indwelling catheters or stents [7].

Infection from indwelling catheters is still thought to be caused primarily by two mechanisms; direct inoculation at time of insertion or ascending movement of bacteria along the low resistance path provided by the catheter. Despite adequate cleansing and preparation, up to 20% of device related UTIs are a result of contamination at the time of insertion [7]. Outside of this the urethral and bladder mucosa possess antibacterial properties and substances to inhibit bacterial invasion [8, 9]. With a catheter in place these mechanisms are bypassed and bacteria travel faster [5] and have an easier route to the bladder, resulting in Catheter-Associated Urinary Tract Infection (CAUTI). Many proven risks for device related UTI have been documented [10, 11] but the main risk factor for CAUTI is indwelling time with a risk of infection between 3% and 7% per day [12].

Behind these high rates of bacteriuria, infection and encrustation in urologic devices is the process of biofilm formation. Biofilms can lead not only to clinically significant infection but also to more rapid development of device encrustation (Fig. 15.1).

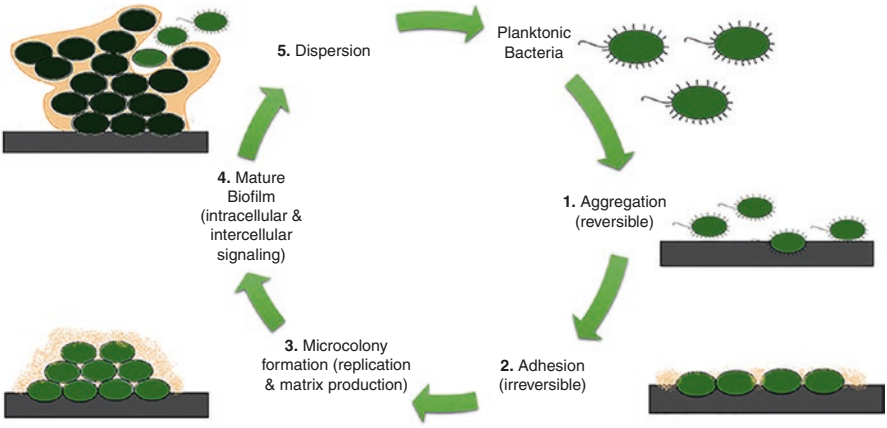

Fig. 15.1 Biofilm formation

Biofilm Formation

A biofilm, by definition, is an aggregate of microorganisms and their extracellular products that form a structured community on a surface. Biofilms are ubiquitous in nature and serve multiple purposes for bacteria including the ability to survive in harsh conditions. They have long been studied, with reports from van Leeuwenhoek in the seventeenth century and later Pasteur in the nineteenth century. For years, the study of planktonic, rather than sessile, bacteria limited the expansion of biofilm knowledge into the medical realm [13]. It was not until the latter half of the twentieth century that the concept was introduced into the medical vocabulary as researchers and clinicians discovered biofilms, first on teeth and then on medical devices. In the urinary tract biofilms are often associated with the typical uropathogenic bacteria*; Escherichia coli, Proteus mirabilis, Enterococcus faecalis, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [14].

The initial step in biofilm formation is development of a conditioning film on the device surface. Within minutes of sterile device insertion into the urinary tract, extracellular components of urine, blood and uroepithelial tissue begin to adsorb to the surface [15–18]. These components can include polysaccharides, ions and glycoproteins among others. Cytokeratins, cell surface proteins on uroepithelial cells, were found to be a major factor in conditioning films on ureteric stents [19]. These researchers also identified blood proteins and inflammatory proteins as contributing causes. Tamm-Horsfall protein, an antimicrobial urinary protein, has been implicated in conditioning films but recent research suggests its role may be limited [20]. Conditioning films alter the surface properties of urologic devices making them more susceptible to bacterial adhesion. As an example, protein components of a conditioning film act as receptor sites for adhesion [21]. Many bacteria lack mechanisms to directly adhere to bare implant surfaces therefore requiring the film as a necessary step in their pathogenesis [22].

The second step in biofilm formation is that of bacterial adhesion to the conditioning film. This results from interactions between the bacteria and their surrounding environment through hydrophobic and electrostatic forces [23]. Bacteria may release photons and signalling molecules to explore their surroundings and identify suitable surfaces for adhesion [13]. Once a surface is located, the initial attachment of bacteria to the conditioning film is reversible. The pattern of attachment, whether in monolayers or clusters, varies between different species of bacteria [13].

What enables bacteria to attach to a surface is the innate expression of outer membrane structures called adhesins. Adhesins include pili, fimbriae, lipopolysaccharide and capsular polysaccharide among others [18]. These provide bacteria with the ability to overcome the often repulsive force from the device surface due to both being negatively charged [24]. The most common uropathogenic bacterium, *Escherichia coli*, utilizes an adhesin know as type I pili to increase its virulence [25]. In contrast, *Klebsiella pneumoniae* requires type 3 fimbriae to colonize its target [26, 27].

The transition from reversible bacterial attachment to irreversible adhesion results from bacterial production of exopolysaccharide or extracellular polymeric substances (EPS) [13]. This constitutes the third step in biofilm formation and marks the transition of bacteria to a sessile state with significantly slowed growth patterns. EPS consists of polysaccharides, proteins, lipids and nucleic acids and functions to provide stability to the microorganisms in the biofilm [28]. It has been estimated that EPS matrix composes up to 90% of a biofilm while cells make up only 10–25% [29]. The matrix contains channels allowing passage of nutrients and oxygen between bacteria [30]. In addition, chemical signalling molecules called autoinducers pass freely between cells in a process termed quorum sensing [31]. These molecules act to control a multitude of physiological activities through changes in gene expression. As a result, bacteria in biofilms acquire mechanisms to evade treatment with antibacterial medications.

Tenke and colleagues summarized these mechanisms of antibiotic resistance succinctly [22]. They include: inability of antibiotics to penetrate through the EPS matrix; internal resistance of the bacteria through genetic changes brought on via quorum sensing; the significant phenotypic differences between planktonic bacteria and the sessile bacteria seen in biofilms; slow growth of biofilm bacteria which reduces the effects of antibiotics that target rapidly growing cells and the ability of bacteria to withstand doses of antibiotics 1000–1500 times the dose that would kill planktonic bacteria of the same species.

The sessile bacteria in a biofilm possess the ability to detach and return to their planktonic state [32]. Some of these microorganisms, such as E coli, produce enzymes to break down the local biofilm matrix [33]. Once released, the planktonic bacteria are able to adhere to a new location on the device, colonize the urothelium or potentially enter the bloodstream [34]. This capacity enables bacteria to transiently cause infection while maintaining an excellent store of sessile bacteria in the biofilm. These infections often respond to initial antibiotic treatment but have high likelihood of recurrence without removal of the indwelling device. The subsequent indolent course of infection is marked by quiescent periods post treatment followed by frequent relapses due to further release of bacteria from the biofilm matrix [35].

Encrustation

In close relationship with biofilms and infection, encrustation of urological devices can prove a challenge to Urologists. Encrusted ureteral stents may lead to sepsis, renal dysfunction from obstruction, multiple operations for removal or even death [36]. For catheters and ureteric stents, the prominent risk factor for encrustation is indwelling time. El Faqih and colleagues showed that less than 10% of stents were encrusted at 6 weeks while over 75% were encrusted by 12 weeks [37]. Similar results were published by Kawahara et al. [38] Other risk factors have been identified including pregnancy, chronic kidney disease, stone disease, metabolic or congenital anomalies, chemotherapy and urinary sepsis [38–40]. Stent characteristics such as size play a role as stents less than 6F encrust more severely than those

greater than 7F [37, 38]. Much research has looked at optimal stent materials, shape, coatings and flow characteristics to minimize encrustation [41–43]. Despite these efforts no currently commercially available stent material or design is able to resist crystal deposition and encrustation [43].

Encrustation occurs from crystalline deposition onto the device surface. Calcium oxalate is the predominant substance while struvite crystals form frequently in infected urine [44]. The process can occur in sterile urine but is often associated with infection by urease producing species [45, 46]. It has been noted that the encrustation of foley catheters is frequently driven by *Proteus* species whereas with ureteric stents *Proteus* is often absent [47, 48].

When urease producing bacteria are present in the urine they stimulate the hydrolysis of urea. This generates ammonia and carbon dioxide which dissolves to form carbonic acid. The excess ammonia results in a decrease in positively charged hydrogen ions and subsequent elevation of the pH. *Proteus* is the most potent urease-producing pathogenic bacteria causing hydrolysis at rates six to ten times faster than urease from other bacteria [23] and is often seen in urethral catheter encrustation [47]. The rise in pH significantly alters the solubility of magnesium ammonium phosphate (struvite) and hydroxyapatite (calcium phosphate) [49]. Calcium and magnesium ions are attracted to the biofilm and begin to crystallize as struvite and calcium phosphate onto the biofilm matrix. In conjuction with this, the biofilm continues to recruit bacteria leading to the self-propagating process of encrustation [47].

In sterile urine, the process of encrustation is much more reliant on patient and device factors than when urease producing bacteria are present. The exact mechanism of encrustation in this setting is still unknown but likely requires biofilm formation on the device surface. The biofilm provides a substrate that attracts urinary crystals and promotes their adhesion [47]. A number of urinary protein constituents have been identified that modulate crystal formation [19, 50, 51]. For patients who are stone formers at baseline, the encrustations on devices typically have the same composition as their other stones [44]. When bacteria are present on ureteral stents, *E. coli* tends be the most frequently isolated species [39]. Whether from infected or sterile urine, encrustation of urologic devices continues to perplex Urologists and prove challenges to researchers working towards the ideal indwelling device.

Outside the Urinary Tract

Outside of the urinary tract Urologists are not free from biofilm formation and subsequent complications. Urologists utilize devices such as artificial urinary sphincters (AUS) and inflatable penile prostheses (IPP) for treatment of urinary incontinence and erectile dysfunction respectively. In a large meta-analysis from 1999 AUS infection rates were 4.7% [52] with more recent estimates around 3% [23]. Infection usually occurs through primary inoculation at the time of implant or urine leak either from urethral injury or erosion. For penile prostheses infection rates in primary implants range from 1% to 3% and increase with more complex

patients [53]. *Staphylococcus* species are implicated in the majority of cases with bacterial seeding occurring at the time of implantation [23]. Infection of either device requires removal of the device and antibiotic treatment. Antibiotics alone are insufficient as they are unable to adequately penetrate the associated biofilm. Unlike with urethral catheters or ureteric stents, removal of these devices is far more involved, requiring general anesthetic. While some Urologists perform simultaneous removal and reimplantation of these devices, the majority will perform two separate procedures [54].

Microbiome

While much is known about the human gut microbiome and other body habitats [55], research into the urinary microbiome is in its early stages. For years the urinary tract was thought to be sterile and no scientific study into a urinary microbiome was even considered. In 2010, Nelson and colleagues, utilizing 16S rRNA polymerase chain reaction (PCR) determined that bacteria were present in male urine specimens that were not detectable by standard urine culture techniques. They found differences in the urinary microbiome in men with sexually transmitted diseases compared to those without [56]. In 2012, with the help of 16S rRNA gene sequencing Wolfe and colleagues determined that bacteria were in fact present in the urinary tract of some women who had negative urine cultures [57]. The same group published further data to support a urinary microbiome in all women, and highlighted the most common species detected [58]. A summary of notable taxa identified in urinary microbiome studies can be found in a review paper by Whiteside and colleagues [59]. Since that time the urinary microbiome has been linked to multiple disease states in urology including interstitial cystitis, chronic prostatitis/chronic pelvic pain syndrome, urge urinary incontinence and recurrent urinary tract infections [60–62].

In regard to encrustation and infection of urologic devices little research exists. It is clear that the microenvironment in the bladder contributes to conditioning film deposition and formation of biofilms [48]. A direct role of the microbiome in this process has yet to be identified. In a study by Azevedo and colleagues, *E. coli* biofilms developed more rapidly in the presence of two non-pathogenic bacteria [63]. This synergistic relationship could lead to increased susceptibility for infection and encrustation in certain patients. Other work by MacLeod and colleagues further highlights the interactions between different bacterial species related to urinary tract infections [64].

A recent study by Tasian et al. highlighted the increased risk of urolithiasis in patients treated with antibiotics [65]. They found that patients exposed to sulfas, fluoroquinolones, cephalosporins, nitrofurantoin/methenamine or broad-spectrum penicillins had an increased odds ratio of stone formation. The risk was highest with early exposure to antibiotics and in those treated within 3–6 months of stone presentation. The risk persisted at 5 years for all categories of antibiotics except penicillins. The authors hypothesize that antibiotic exposure places selective pressure on the urinary microbiome which may lead to intensified crystallization. Work done by

Dethlefsen and colleagues looking at the gut microbiome adds strength to this argument [66]. They examined the microbiome in patients subjected to antibiotics and found that bacterial diversity decreased within 3–4 days after exposure. It wasn't until at least 1 week following cessation of treatment that the microbiome began its return to a pre-exposure state. Within months after exposure the gut microbiome had stabilized but never returned to its initial state. Changes similar to these could contribute to destabilization of the urinary microbiome, leading to increased risk for biofilm formation, infection and encrustation of devices.

Conclusion

Encrustation and infection of urologic devices continues to challenge practitioners and burden the healthcare system. Biofilm formation increases the likelihood of device related complications and impedes effective treatment. Though much work is underway to improve devices and limit biofilm formation, no device, coating or material has proved successful to this point. As researchers delve deeper into the urinary microbiome answers around biofilm formation, infection and encrustation may surface.

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The Role of Bacteria in Non-infection Stone Formation

16

Ryan Dornbier, Petar Bajic, Andrew Schwaderer, and Alan J. Wolfe

Introduction

Urinary stone disease (USD) is frequently encountered in United States healthcare with an estimated lifetime prevalence of 10.6% in men and 7.1% in women [1]. The prevalence of urinary stone disease has doubled over the last 15 years, with increases more pronounced in historically less affected groups, such as children and women [1, 2]. Moreover, the risk of recurrence is significant with 39% of first time stone formers having a second episode within 15 years of follow up [3]. The economic burden of USD in the United States is immense, resulting in over 600,000 emergency room visits and $2 billion in annual expenditures [4].

The role of bacteria in USD has historically been limited to the association between urease-splitting organisms and magnesium-ammonium-phosphate (struvite) stones, as discussed in previous chapters. However, infection stones make up only 4% of stones with calcium-based stones (calcium oxalate (CaOx) and calcium

R. Dornbier · P. Bajic
Department of Urology, Loyola University Medical Center, Maywood, IL, USA
e-mail: ryan.dornbier@lumc.edu; pbajic@lumc.edu

A. Schwaderer (✉)
Department of Pediatrics, Division of Nephrology, Indiana University, Indianapolis, IN, USA

Riley Physicians Kidney Diseases, Indianapolis, IN, USA
e-mail: schwadea@iu.edu

A. J. Wolfe (✉)
Department of Microbiology and Immunology, Loyola University Chicago, Maywood, IL, USA

Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Center for Translational Research and Education, Maywood, IL, USA
e-mail: awolfe@luc.edu

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phosphate (CaPhos)) constituting the majority. Other more common stones include uric acid and mixed composition stones [5]. The role of bacteria in non-infection stone disease has not been well defined, but mounting evidence indicates that bacteria may play an important role.

Evidence of Bacteria in Non-infection Urinary Stone Disease

Urinary Tract Infection and Urinary Stone Disease

The concurrent presentation of urinary tract infections with USD for both obstructing and non-obstructing stones is a common clinical occurrence. In a cohort of 1325 Scandinavian patients with USD, 28% had a positive standard urine culture. Of the 535 patients with calculi available for analysis, 31% had a positive standard urine culture at the time of presentation, regardless of stone composition [6]. This association was true in Japanese patients as well; 7% of stone forming patients had a positive standard urine culture within 1 month of surgical intervention [7]. In the pediatric population of Taiwan with newly diagnosed USD, the most commonly associated condition was a history of urinary tract infection at 34.1% (23.5% of males, 43.9% of females) [8].

Outside of clinical presentation and association, many patients with recurrent UTIs have resolution of bacteriuria on standard urine culture after stone removal. In an analysis of 120 patients with recurrent UTI and asymptomatic, non-obstructing renal calculi, Omar and colleagues found that 48% of patients were infection free at an average follow up of 14 months. The majority of stone compositions were calcium-based, while only 6 (5%) patients had struvite stones [9]. Oliver and colleagues looked at a similar cohort of 103 patients with positive preoperative standard urine culture (79%) or recurrent UTIs with negative preoperative standard urine culture (21%). Following ureteroscopy, 70.7% of patients were infection-free at 12 month with most stones (74%) being composed of calcium oxalate. Moreover, 80% of patients with stone recurrence also had recurrent infections, suggesting an association between the recurrent infection and the recurrent stone [10].

Infectious complications are a known hazard of USD management for all stone compositions. The American Urological Association and European Association of Urology recommend the routine use of preoperative standard urine culture and prophylactic antibiotics prior to any surgical stone manipulation [11, 12]. Despite this practice, sepsis occurs in 4.7% of patients undergoing percutaneous nephrolithotomy (PCNL) [13]. In reviews from the Endourological Society, 8.8% of patients with negative preoperative standard urine culture undergoing PCNL developed fever [14]. Though infectious complications are more commonly associated with infection stones [15], the development of systemic inflammatory response syndrome (SIRS) has been demonstrated in up to 5.3% of patients with non-infection stones [16]. Similarly, Rivera and colleagues reviewed their experience in 227 patients undergoing PCNL for management of USD; infectious complications (UTI/

SIRS/Fever/Sepsis) occurred in 37 patients (16%). Overall, 73% of patients experiencing infectious complications had non-struvite stone composition [17].

Bacteria Can Be Cultured from Non-infection Stones

In addition to the association with UTI and infectious complications, multiple studies have demonstrated the ability to culture bacteria from urinary stones (Table 16.1) [18–25]. Depending on the study, bacteria have been isolated from urinary stones in 7% to 75% of stone cultures. When limiting to non-infection stones, positive culture results have been obtained in 5–33% of stones. Pure calcium stones (CaOx or CaPhos) are culture positive in up to 44% of cases. Moreover, these stone cultures contain bacterial isolates of both non-urease splitting and urease splitting organisms. Figure 16.1 [18, 21, 26, 27] demonstrates the frequency of primary isolates from 455 positive urinary stone cultures. Commonly implicated urease splitting bacterial genera include *Staphylococcus, Proteus, Klebsiella, Pseudomonas,* and *Providencia.* However, non-urease splitting bacteria also have been isolated, including known uropathogens *Escherichia coli* and *Enterococcus spp.*

Enhanced Culture Techniques and 16S rRNA Sequencing of Urinary Stones

The previous studies relied on culture protocols similar to techniques popularized by Stamey and colleagues in the 1970s [25]. These culture protocols involve washing the stones in saline and crushing, prior to plating on standard culture media. However, these protocols are not designed to isolate the slow growing, fastidious organisms that make up a majority of urinary biomass [28]. The recent use of enhanced culture-based methods, such as enhanced quantitative urine culture (EQUC), and culture-independent methods, such as 16S rRNA gene sequencing, have demonstrated the existence of resident microbes in the urinary bladder (called the urinary microbiome or urobiome) and debunked the historical view that the bladders of women and men are sterile [28–31]. EQUC utilizes increased urine volumes, longer incubation time, multiple media types, and a variety of atmospheric conditions to isolate slower growing bacteria [29]. 16S rRNA sequencing allows for the identification of bacteria that cannot be cultured (e.g., those exposed to antibiotics prior to collection). In initial experiments utilizing 16S rRNA sequencing on five kidney stones from calcium oxalate stone-formers, our group identified members of several bacterial taxa, including *Pseudomonas*, *Gardnerella*, *Lactobacillus*, *Enterobacteriaceae*, *Bradyrhizobium*, *Phyllobacterium*, and *Brucella.* As stones represent a low biomass medium, available in limited quantities, strategies to determine which sequenced bacteria are truly stone associated are ongoing. The incorporation of EQC, a stone-relevant derivative of EQUC, allowed for the isolation of live *Pseudomonas* and *E. coli* strains in two of the stones collected. In each case, this was concordant with the dominant organism

Table 16.1 Percent of urinary stones with positive culture growth, overall and subdivided by stone composition

Study (n = stones analyzed)	Positive stone culture (%)	CaOx (%)	CaPhos (%)	CaOx + CaPhos (%)	Calcium + Uric acid (%)	Uric acid (%)	Non-infection stones (%)	Any infection comp. (%)
Paonessa et al. (n = 776) [18]	38.7	NA	NA	NA	NA	NA	32.7	69.6
Wang et al. (n = 32) [19]	18.8	18.8	NA	NA	NA	NA	18.8	NA
Shafi et al. (n = 45) [20]	22.2	14.3	NA	NA	0.0	0.0	8.8	63.6
Tavichakorntrakool et al. (n = 100) [21]	36.0	13.3	0.0	35.3	75.0	35.7	32.9	53.3
Golechha et al. (n = 100) [22]	31.0	16.7	20.0	15.7	NA	NA	16.2	73.1
Gault et al. (n = 258) [23]	7.0	1.2	14.3	NA	NA	5.3	5.4	16.2
Dajani et al. (n = 130) [24]	15.4	12.5	16.0	NA	NA	29.4	15.4	NA
Thompson et al. (n = 86) [25]	76.7	44.0	16.7	NA	NA	0.0	33.3	95.9

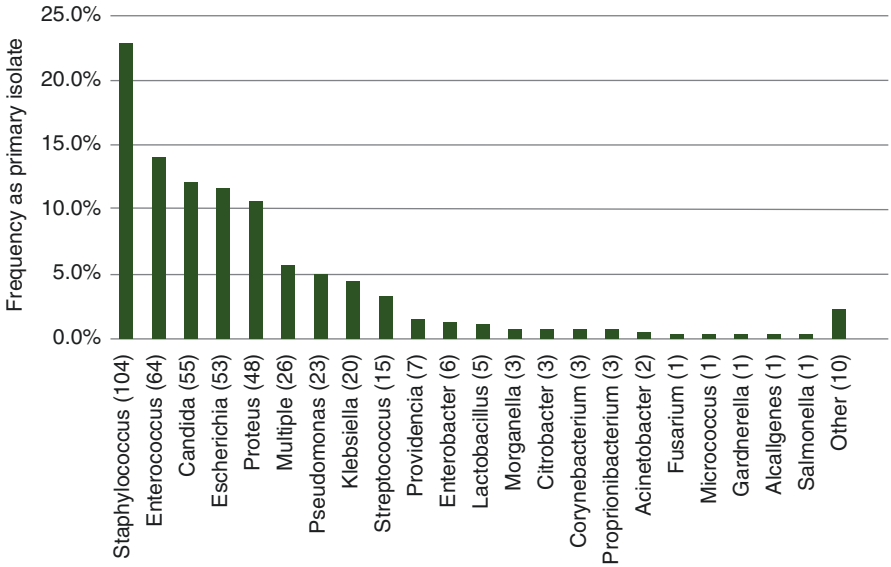

Fig. 16.1 Frequency of bacterial isolates from positive urinary stone culture (Stone samples = 1283; positive culture = 455) [18, 21, 26, 27]

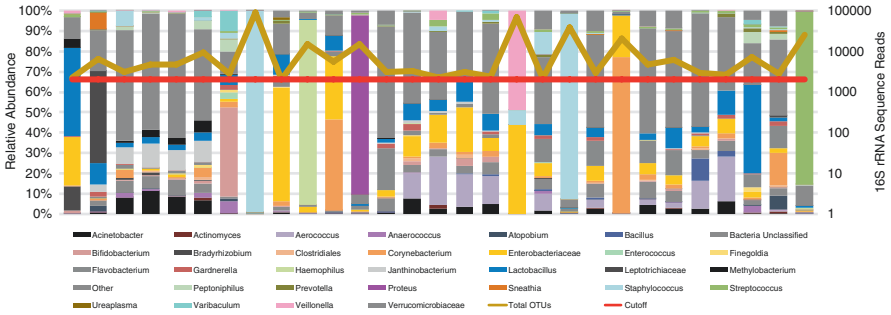

Fig. 16.2 16S rRNA gene sequencing of 29 sequence positive stones obtained via ureteroscopy

identified by 16S sequencing [32]. Analysis of 52 additional kidney stones obtained via ureteroscopy identified 29 (55.7%) sequence positive stones (Fig. 16.2). 16/29 (55%) were composed entirely of non-infection stone compositions, while only one stone contained elements of struvite. Furthermore, EQC was able to isolate bacteria from 11/29 (37.9%) of sequenced stones [33]. These results reflect the idea that live bacteria are associated with non-infection stones, regardless of antibiotic exposure.

Contribution of Bacteria to Urinary Stone Disease

It is now clear that bacteria are associated with stones of all compositions. Patients with USD often have concomitant UTI or infectious complications following intervention, and bacteria are readily sequenced and cultured from stone samples. Though the presence of bacteria in non-infection stones is apparent, it is unclear whether a causal relationship exists.

Supersaturation and Bacteria as a Modifier of Urinary Composition

Supersaturation of urinary solutes has long been recognized as a major pathophysiologic factor in USD. As the concentration of urinary components, mainly calcium and oxalate, reach their limits of solubility, crystallization can occur resulting in stone formation [34, 35]. Historically, the understanding of bacterial contribution to urinary solutes has focused on the intestinal microbiota; specifically, the role of *Oxalobacter formigenes*, which metabolizes dietary oxalate, reducing oxalate concentration of the urine, and providing a protective effect for recurrent CaOx stone formation [36, 37]. The role of *O. formigenes* in CaOx stone formation is discussed in depth in other chapters; notably, however, its role as a probiotic to protect against CaOx stone formation has been of limited success [38–40]. *Oxalobacter* does not commonly inhabit the genitourinary tract. However, other oxalate-degrading bacteria have been identified in the mammalian gut including *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, and *Streptococcus*; microbiota that are more routinely isolated from the genitourinary tract [41]. The role of these bacteria in the urine is not currently understood.

Outside of oxalate-degrading bacteria, the gut microbiome appears to have a complex relationship with urinary solute concentration. Stern and colleagues, investigated the gut microbiome of 11 stone formers and their 24 h urine collection. They found an inverse relationship between *Escherichia* and urinary citrate; as well as an inverse relationship between *Eubacterium* and urinary oxalate [42].

Though the relationship is complex, the gut microbiome may have a protective role in USD formation. However, as previously discussed, urinary bacteria appear to be strongly associated with urinary stone formation. Hypocitraturia is a known risk factor for calcium stone formation. Urinary citrate is a strong inhibitor of stone formation as it binds to free calcium, reducing urinary calcium concentration, making urinary calcium less available to complex with oxalate [34]. Urinary bacteria may contribute to stone formation by metabolizing citrate, lowering urinary citrate concentration, thereby promoting calcium oxalate supersaturation and urinary crystal formation. In a study of idiopathic calcium stone formers, De Ferrari and colleagues noted significantly decreased urinary citrate concentrations in 17 urine culture positive patients compared to standard urine culture negative patients. These standard urine cultures grew *Escherichia*, *Streptococcus*, *Staphylococcus*, *Pseudomonas* and *Citrobacter* species [43]. Moreover, using in vitro urinary models, *E. coli*, both pathogenic and non-pathogenic, decreases urine citrate levels, promoting crystallization [44, 45].

Bacteria as Crystal Aggregator

Bacterial modification of urinary solute concentrations potentiates known risk factors of nephrolithiasis. However, supersaturation of urinary solutes alone does not always result in USD, as there is considerable variation in urine chemistries between stone formers and non-stone formers [46, 47]. As such, bacteria may play a role in crystal adherence, acting as a nidus and promoting crystal deposition in patients with stone solutes. Using an in vivo murine model, our group induced a CaOx nephropathy and uropathogenic *E. coli* (UPEC) pyelonephritis in ten mice. There was a significantly higher number of CaOx deposits in the CaOx and UPEC inoculated mice compared to CaOx or UPEC inoculated mice alone [32]. Similarly, crystals have been shown to aggregate on both Gram-negative and Gram-positive bacteria. Chutipongtanate and colleagues analyzed strains of *E. coli*, *K. pneumoniae*, *S. aureus*, and *S. pneumoniae*, finding increased CaOx crystal growth and aggregation with all four bacteria compared to controls [48].

There also is the possibility that bacteria may play an indirect role in crystal aggregation. In addition to crystals, urinary stones contain a protein matrix that frequently contains innate immune proteins [49]. Urinary stones create an inflammatory response that results in the release of inflammatory proteins and cytokines. This promotes the growth and adhesion of CaOx and uric acid crystals [50–53]. In our study, mice inoculated with CaOx and UPEC had an increased expression of inflammatory and stone matrix protein genes compared to inoculation with either substance alone [32]. Therefore, it is reasonable to believe that the presence of bacteria may work synergistically with CaOx to potentiate stone formation and aggregation. This is similar to findings in vascular calcifications in which bacteria potentiate atherosclerotic plaque formation [54–56].

Conclusion

The role of bacteria in non-infection USD is complex and poorly understood relative to that of infection stones, but the association of bacteria with all stone compositions is undeniable. Patients with non-infection stones present with UTIs, experience infectious complications after stone procedures despite negative standard urine culture, and have positive stone cultures. The use of 16S rRNA sequencing and enhanced culture techniques has expanded our knowledge of stone-associated bacteria and allowed for isolation of these bacteria. The mechanism by which bacteria promote stone formation in non-infection stone formation is an area of active research. Going forward, it is important that we further investigate this association by sequencing and isolating a larger number of stone bacteria from a diverse patient population, evaluating the genomic and proteomic capacities of these bacterial isolates, and determining their effect on stone contribution via in vitro and in vivo models.

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The Role of the Intestinal Microbiome in Oxalate Homeostasis

17

Aaron W. Miller

Introduction

Oxalate is a simple, two-carbon organic acid consumed widely in foods such as nuts, berries, spinach, and potatoes [1, 2]. In addition to dietary intake, oxalate is also produced endogenously as a terminal metabolite in the liver, from precursors such as glycine, phenylalanine, some pentose sugars, hydroxyproline, and glyoxal among others [3]. Of these compounds, hydroxyproline and glyoxal are the most prominent contributors to endogenous oxalate [3]. Primary hyperoxaluria, defined by a group of three autosomal recessive disorders, affects approximately one out every 120,000 individuals and leads to excess excretion of urinary oxalate resulting from increased endogenous production [4–6]. In individuals without primary hyperoxaluria, the contribution of dietary oxalate to urinary oxalate excretion has been estimated between 24.4% and 52.6%, dependent upon the amount of oxalate and calcium consumed, with the remainder of excreted urinary oxalate being composed of endogenous sources [1]. In laboratory rodents, increasing dietary calcium from 0.01% to 1.2% can decrease urinary oxalate excretion 15-fold [7]. Conversely, increasing dietary oxalate in laboratory rodents from 0% to 1.5%, drives a fivefold increase in urinary oxalate excretion [8]. Hyperoxaluria, or high urinary oxalate excretion, is a known risk factor for USD regardless of the source of the compound [9, 10], with transient, diet-induced spikes in oxalate concentration potentially playing an important role in calcium oxalate stone growth [11, 12]. Thus, to reduce the risk of recurrent calcium oxalate stone disease, it is imperative to understand the factors that contribute to a homeostatic environment relative to oxalate.

A. W. Miller (✉)
Department of Urology, Lerner Research Institute, Glickman Urological and Kidney Institute, Cleveland Clinic, Cleveland, OH, USA
e-mail: millera25@ccf.org

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Numerous studies have reported the existence of diverse oxalate-degrading bacteria from human and other mammalian sources, thus giving rise to the hypothesis that oxalate-degrading bacteria in the gut contribute to oxalate homeostasis and reduces the risk for USD [13–19]. While diverse oxalate-degrading bacteria have been isolated, much of the research has focused on a single species, *Oxalobacter formigenes*, which requires oxalate as a carbon and energy source for growth. Collectively, 81% of clinical investigations (9 out of 11) report a significant difference in colonization by *O. formigenes* between patients with a history of USD compared to healthy controls [20]. However, the rate of colonization ranges from 11% to 100% for the healthy population and 0–100% for the USD population [20]. Furthermore, only 55% of studies (7 out of 13) report an effect of colonization by *O. formigenes* on urinary oxalate [20]. Collectively, these data indicate that *O. formigenes* colonization alone is neither necessary nor sufficient to prevent USD or to lower urinary oxalate. Instead, recent metagenomic studies suggest that oxalate metabolism and excretion is influenced more from a cohesive community of bacteria rather than oxalate-degrading bacteria alone [8, 21–23].

Identification of an Oxalate-Degrading Microbial Network

To date, two primary methods have been developed to identify the oxalate-degrading microbial network from the gut microbiota. The first method involves stimulating the oxalate-degrading microbial network with dietary oxalate, which assumes that if a bacterial population is associated with or benefits from oxalate exposure, the population will expand with increasing oxalate exposure. The white-throated woodrat, *Neotoma albigula*, is a wild mammalian rodent that consumes a high oxalate diet in the wild and harbors a gut microbiota that degrades nearly all dietary oxalate consumed [17, 21, 23–25]. Thus, it is ideally suited for studies on the interaction between the gut microbiota and dietary oxalate. When dietary oxalate is gradually increased for these animals, there is a net increase in total microbial diversity in the gut with no significant reduction in abundance for any taxa, indicative of a broad beneficial impact of oxalate on the microbiota [21, 23]. When fecal transplants are given from *N. albigula* to laboratory rodents, there is a significantly greater reduction in urinary oxalate than when oxalate-degrading bacteria are administered alone, confirming the hypothesis that oxalate metabolism and excretion is regulated by a community of bacteria rather than isolated species [8]. The oxalate exposure method to identify the oxalate-degrading microbial network is conservative and only detects bacteria that explicitly exhibit a positive response to oxalate.

The second method developed to identify the oxalate-degrading microbial network looks for bacteria in the gut whose relative abundance correlates to *O. formigenes* [8, 26–28]. This method of identification relies on the assumption that *O. formigenes*, as a species that requires oxalate as a growth substrate, is at the center of any oxalate-degrading microbial network and that other members of the network will exhibit a relative abundance similar to *O. formigenes*. Association with *O.*

formigenes is a less conservative method of identifying the oxalate-degrading microbial network as it requires more assumptions and there is no direct association with oxalate. However, this method can be done using a single snapshot of the microbiome and does not require exposing subjects to a load of oxalate, thus making it ideal for clinical studies. Despite the differences between the two techniques, the network of bacteria detected by the two methods largely overlap (Fig. 17.1, panel 1) [8, 21–23, 26–28].

The mechanisms of how oxalate is processed within the oxalate-degrading microbial network is currently unknown, but has been speculated upon. Specifically, oxalate metabolism results in the by-products of CO_2 and formate, both of which can be utilized in acetogenic, methanogenic, and sulfate-reducing pathways [29–31]. Thus, in the context of a complex microbial community, such as that found in the gut, the by-products of oxalate metabolism may be used in these metabolic pathways downstream [21, 23]. In fact, acetogenic, methanogenic, and sulfate-reducing bacteria, from genera such as *Clostridium*, *Oscillospira*, *Odoribacter*, and *Desulfovibrio*, are typically included in oxalate-degrading microbial network analyses (Fig. 17.1, panel 1) [8, 21–23, 26, 27]. Even within isolated species, oxalate metabolism is often linked with acetogenesis and formate metabolism [32–35].

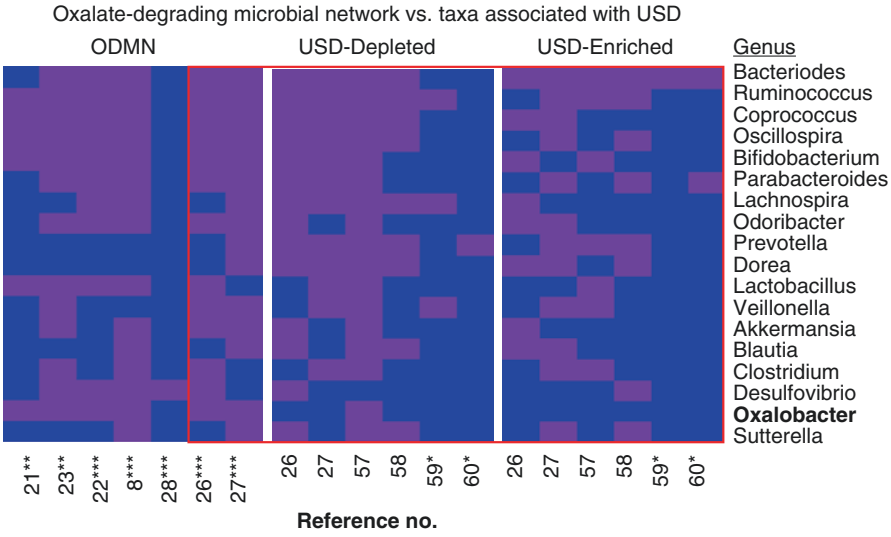

Fig. 17.1 Meta-analysis of all studies that have examined the whole gut microbiota in terms of the OTUs associated with oxalate metabolism or enriched/depleted in the USD population, summarized to genus-level taxonomy. Genera are ordered from those that are enriched the most often (top) to least often (bottom). Primary data sources were independently analyzed if available. Red box indicate clinical studies. *ODMN* Oxalatedegrading microbial network. *Primary data not available. **Identification of ODMN through diet trials. ***Identification of ODMN through association with *O. formigenes*

Relevance of the Oxalate-Degrading Microbial Network to USD

In studies that have used culture-based methods of *O. formigenes* detection or PCR-based methods using *Oxalobacter formigenes* specific primers, 81% have found that there is a significant negative correlation between *O. formigenes* colonization and USD with highly variable rates of colonization between healthy and USD populations [20]. Several studies have also shown a negative correlation between antibiotic use and *O. formigenes* colonization, providing a potential mechanistic link between antibiotic use and the onset of USD [36–39].

Given the negative association between USD and oxalate-degrading bacteria such as *O. formigenes*, several studies have explored the use of oral probiotics to reduce urinary oxalate in both human and animal models. In animal studies, oral administration of *O. formigenes* leads to a significant reduction in urinary oxalate [7, 40–43]. However, colonization by *O. formigenes* is typically transient. In individuals without detectable oxalate-degrading activity in the stool or with idiopathic hyperoxaluria, administration of *O. formigenes* reduced urinary oxalate levels [44, 45]. However, *O. formigenes* colonization was transient in one of the studies [45]. For individuals with primary hyperoxaluria, administration of *O. formigenes* has not led to similar reductions in urinary oxalate excretion in Phase II/III trials despite promising animal and Phase I clinical studies [7, 42, 43, 46–49]. Similar significant but transient reductions in urinary oxalate have been reported after oral administration of oxalate-degrading lactic acid bacteria, in both animal and human trials [16, 50–56].

In recent metagenomic studies, only 16% have found a significant reduction in *O. formigenes* in the USD population (Fig. 17.1, panel 2) [26, 27, 57–60]. The discrepancy between metagenomic studies and previous culture- or PCR-based studies is unknown. However, two independent metagenomic studies found that USD patients harbored a lower diversity of oxalate-degrading bacteria overall, even though there was no difference in colonization by *O. formigenes* [57, 60]. Furthermore, a recent clinical study has found that patients with an active episode of USD were significantly more likely to have taken antibiotics in the last year compared to subjects with no history of USD [26]. However, in contrast to previous culture- and PCR-based studies, there was no negative association between antibiotics and *O. formigenes* colonization. The association between antibiotics and USD corroborate another retrospective clinical study [61]. Collectively, metagenomic studies associated with the oxalate-degrading microbial network or USD show that the bacterial genera depleted from the gut microbiota of the USD population strongly overlap with the genera associated with the oxalate-degrading microbial network, suggesting that this network rather than isolated species may help to prevent USD (Fig. 17.1) [8, 21–23, 26, 27, 57–60]. Further evidence that the oxalate-degrading microbial network may help to prevent USD comes from recent animal studies, which found that cefazolin, an antibiotic commonly used prior to surgery for stone removal, leads to a persistent increase in urinary oxalate specifically due to its effect on the microbiome [62].

Conclusions and Implications for Urological Practice

Hyperoxaluria is a known risk factor for the development of the most common stone type, calcium oxalate [9, 10]. Several factors are known to influence oxalate homeostasis and excretion. These include the levels of oxalate and calcium consumed [1, 7, 8], the presence of endogenous oxalate precursors [3], host genes [4–6], the fat and sugar content of the diet [62–65], gut microbiota composition [8, 22, 57, 60], and antibiotic use [62]. Metagenomic and fecal transplant studies reveal that the contribution of the gut microbiota to oxalate homeostasis revolves around diverse oxalate-degrading species along with other bacteria that may be indirectly benefitting from oxalate exposure [8, 17, 21–23, 57, 60]. Clinical studies show that individuals with USD are associated more with a reduction in the network of bacteria surrounding oxalate metabolism rather than a reduction in a single species of oxalate-degrading bacteria (Fig. 17.1). Furthermore, clinical and animal studies reveal a link between oral antibiotics and USD or urinary oxalate, respectively [26, 61, 62]. However, more work is needed to determine if oral antibiotics, which are commonly prescribed prior to surgical procedures for stone removal, impact oxalate homeostasis in a clinical setting. Regardless, results are clear that to effectively mitigate oxalate excretion, the composition of a patient's gut microbiota must be considered in addition to the diet and other factors.

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