Dirk Lange
Ben Chew *Editors*

The Role of Bacteria in Urology

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Chapter 1
Bacteria in the Genitourinary Tract: The Microbiota and Probiotics

Gregor Reid

Abstract The identification of an array of bacterial species in the urinary tract, detected by DNA sequencing, has the potential to change many aspects of urological practice. If they are associated with health or disease, should all urines be sampled as part of patient management, and what is the consequence of antibiotic therapy? Can an aberrant microbiota be manipulated by probiotics, drugs or diet resulting in less risk or better control of disease? To answer these questions, more microbiome studies are needed along with methods that interpret the data in a clinically relevant manner. Cause and effect remains to be established in most cases, but this area has the potential to invigorate urological research and improve patient care in the not so distant future.

Introduction

It was not long ago that bacteria were regarded in Urology as being pathogenic agents causing infection or organisms used for treatment of superficial bladder cancer. However, the recent discovery of an array of bacteria in the urinary tract of apparently healthy subjects is changing how we view these microbes.

The term microbiota refers to the microorganisms of a particular site, habitat, or geological period, and in the case of urology, those recovered from urine or a tissue site. The term microbiome has a wider context referring to the ecological community of all microorganisms and their genes and genomes that literally share our body space. The urinary microbiome therefore refers to all the organisms (microbiota) and their genomic activities. It is rather semantic given that microbiota without genes would not exist! In this chapter, microbiota will be used.

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The existence of a microbiota in the distal urethra, vagina, prepuce, external genitalia has been known for a long time, mainly as a source of organisms that can infect the urinary tract. It is deemed 'normal' if it does not cause infection or inflammation, but the term is not ideal. In most cases, bacteria exist at these sites with genetic elements that could induce infection and/or inflammation, thus it is not an all-or-nothing situation. Rather, a 'normal' microbiota would be one that is normal or homeostatic for the individual when he/she is healthy. In general, the most abundant organisms would be non-pathogenic, for example comprising *Lactobacillus* in the adult vagina.

In the following sections, the role that the 'normal' microbiota play in urinary tract health will be discussed, along with efforts to supplement or re-set the microbiota using probiotics.

Why Is There a Microbiota in the Urinary Tract?

It is relatively simple to understand how bacteria enter the urinary tract, as the site is open to a microbial environment. In terms of why they are present, the long-standing theory has been that certain species and genera have adapted to the urinary environment and have formed a barrier against disease. The mechanisms include maintenance of epithelial and mucosal integrity, priming of the innate and adaptive immune systems, countering pathogens through excluding them from adhering and growing at the site, helping to control pH, degrading or reducing toxic compounds, and likely through signalling processes helping the overall host's defences [1, 2]. These constitute a remarkable number of ways in which bacteria are beneficial to urological health, yet the power of these effects has not been propagated to the extent that it could be. Rather, the unfruitful search for vaccines against adhesion to the uroepithelium by pathogens, the narrow-minded design and improper use of broad spectrum antibiotics, the attempt to use compounds to bind and flush out pathogens, and the modest at best effects of cranberry juice [3–7] have not ablated the suffering of hundreds of millions of women worldwide, from urinary tract infection (UTI) nor led to new management options.

If bacteria have deliberately found residence in the urinary tract as part of evolution, one would expect the effects to be symbiotic. For the organisms, a niche with nutrients is an obvious benefit, but what of the host? Given that it has taken sophisticated DNA sequencing to detect a urinary microbiota [8–11], and the organisms are therefore not flourishing in large numbers, presumably they are not very metabolically active. Studies of *Mycobacterium tuberculosis* have shown that a portion of quiescent bacteria is indeed metabolically active [12], so the same may be true for some members of the urinary microbiota. While purely speculative, the microbiota may be kept quiescent by innate and adaptive host defences, but activity might increase at different times in response to changes in nutrient content, hormones, lowered immune status, or other factors in the urine [13, 14]. These activations might lead to host responses and/or signaling molecules affecting the nervous system [15, 16], resulting in mild symptoms and signs of discomfort. Indeed, such presentations are not uncommon, often verging on being completely asymptomatic.

If bacteria are sparsely distributed around the bladder, in some cases internalized or in small biofilms, could they be helpful to reduce discomfort? It is known that some of the species detected [17], especially *Lactobacillus* [18] can produce neuroactive compounds including some that can potentially reduce pain. An alteration in the composition of the microbiota, such as an increase in *Lactobacillus gasseri* and decrease in *L. crispatus*, might not only induce urgency incontinence [17], but it may trigger other perceptions of discomfort. The necessity for treatment, in most cases with pharmaceutical agents, could in fact make the situation worse through altering the microbiota to a more aberrant one [19, 20]. So, what options remain for managing urinary dysfunction?

What Happens When the Microbiota Is Disrupted?

The administration of antibiotic therapy is often used not just to treat proven infection, but also to try and prevent recurrences, or in an attempt to reduce symptoms and signs that are otherwise unexplained. These are being questioned primarily because of side effects and antibiotic resistance [21–24], but they may also have consequences for later obesity and its complications [25]. The development and use of broad spectrum antibiotics was somewhat well intentioned, to rapidly provide relief from symptoms of UTI without waiting for culture results. But, on reflection it was poorly conceived. A much better approach would have been to develop agents that specifically and only targeted the offending organism, in the case of UTI mostly *E. coli.* With few, if any, new antibiotics in the pipeline, and companies reluctant to invest in such research, assuming such agents could even be developed, we are left with needing to carefully assess when to use the current armamentarium and when not to. Options such as relieving pain and using single dose therapy are worthy of consideration [26, 27], albeit with careful follow-up to ensure no precipitation of the infectious process.

The option of administering beneficial bacteria to out-compete pathogenic ones was first considered in the early 1980s [28], but not taken seriously by the medical community until much later. The initial approach was to interrupt the ascension of uropathogens from the vagina and perineum to the urethra and bladder, by instilling lactobacilli with properties that could interfere with pathogen growth and adhesion [29]. Then, direct instillation of lactobacilli into the bladder was attempted but the organisms did not appear to colonize [30, 31]. However, in the late 1980s no DNA sequencing methods were available and the urinary microbiota had not been discovered, so it is possible that this approach had more validity than was realised at the time. Subsequent attempts to colonize the bladder with avirulent *E. coli* have met with some success [32], although some patients complained of foul odor, and scaling up to widespread use will face many challenges. In neurogenic bladder patients, this could prove to be a useful therapy, and how the *E. coli* HU2117 strain interacts with the microbiota of responders and non-responders would be worthwhile investigating.

The discovery of uropathogens that persist in and on the uroepithelium, and others that form internalized dense pods, often as a reaction to antibiotics [33, 34], also has consequences for management. Antibiotic therapy disrupts the microbiota

composition for months [35, 36], without necessarily eradicating the offending pathogens. The consequences may be temporary eradication of symptoms and signs of infection, but due to pathogen persistence and microbiota disruption, an increased risk of longer term recurrences and complications. This could be particularly problematic for pregnancy where UTI is a preventable cause of maternal and neonatal morbidity and mortality [37], and where antibiotic use might make an initial impact on the UTI, but lead to other later complications.

The ability of lactobacilli to penetrate and disrupt pathogenic biofilms has been shown [38, 39], and may be particularly important if it re-sets the microbiota to homeostasis. In cases where probiotic lactobacilli have been effective at preventing UTI in children with reflux [40] and adult women [41–43], the use of probiotics to prevent infection and possibly recurrence of bladder cancer have merit.

How Does This Knowledge Change Urological Practice?

Urinary tract diseases continue to adversely affect quality of life of many people [44]. Until suitable alternative, clinically proven therapies are made available, the only options for urologists remain surgery and pharmaceutical therapy, or possibly use of probiotics. Still, there are critical messages that warrant consideration from the latest research on microorganisms discussed herein:

- Awareness of a microbiota in the urinary tract means that unexplained conditions, or ones with no known cause, might involve bacteria, in which case performing DNA analysis of urine and tissue could uncover such a link. Such methodologies will become available, not just in research-oriented institutions, albeit at a cost.
- The administration of antibiotics, especially for prophylaxis at surgery and in children with vesicoureteral reflux could have serious ramifications in the patient's future.
- A holistic approach is recommended, especially when 'traditional' therapies fail. This might include use of anti-inflammatory or anti-pain medication, although the impact of these on and with the microbiota must be considered [45].
- Probiotic strains that have been clinically documented, such as L. rhamnosus GR-1 and L. reuteri RC-14, should be considered to prevent infection and improve treatment of infection and possibly cancer, as well as reduce side effects of drug therapy.
- Studies are needed to understand the role of human and bacterial viruses in urinary tract health.

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Chapter 2
Overview of Urinary Tract Infections

Joey Lo, Way Ho Choi, Justin Y.H. Chan, and Dirk Lange

Abstract For the longest time both urinary tract and urine were thought of as sterile, however recent evidence seems to suggest the presence of a urinary tract microbiome, believed to play a significant role in maintaining overall urinary health [1]. Similarly to the intestinal microbiome, the urinary tract microbiome likely plays a role in keeping uropathogens at bay, preventing them from getting the upper hand. When they do take over any part of the urinary tract, patients will develop a urinary tract infection (UTIs). Depending on the location along the urinary tract, infections are classified into upper or lower UTIs. Lower UTIs generally involve the bladder (called cystitis), urethra (urethritis) and prostate (prostatitis) while upper UTIs involve the kidneys and are also considered ascending infections due to the bacteria ascending from the bladder to the kidneys. Here we provide an overview of each type of UTI and discuss some of the most common pathogens involved along with a brief discussion about the most common pathogenic mechanisms for each.

Cystitis

Cystitis is the presence of bacteria confined to the urinary bladder [2], characterized by dysuria, frequency, urgency, cloudy urine, with or without suprapubic pain [2–5]. It is often associated with pyuria, and occasionally with haematuria [2]. The infection can be classified as either uncomplicated or complicated, where uncomplicated cystitis refers to cases where the host is healthy with no structural or functional

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abnormalities, and is neither pregnant nor has indwelling devices medical devices such as urinary stents or catheters. All cystitis cases that do not fit these criteria are considered complicated [4]. Approximately 95 % of all urinary tract infections (UTIs) are uncomplicated bladder infections, which will be the focus of this section [6].

Acute, uncomplicated cystitis (AUC) occurs in both men and women, but is primarily present in young, sexually active women, 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]. Occasionally, other Enterobacteriaceae, such as *Proteus mirabilis* and *Klebsiella* spp., or Enterococci are isolated [3, 10]. 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 and *S. saprophyticus* 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 [11]. Once inside the bladder, uropathogens find ways to adhere to and infect uroepithelial cells, or they become internalized by host cells where they proliferate while hiding 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, hair-like fibres which are distributed throughout the bacterial surface [6]. This particular structure is formed by two adapter proteins, known as FimF and FimG, along with a mannose-binding adhesin, FimH. FimH mediates bacterial adherence to many host glycoproteins and non-glycosylated peptide epitopes, and is both necessary and sufficient for the initiation of the invasion process which leads to the internalization of the bound bacterium into the host cell. Once internalized, the UPEC can use the host cells as a protected niche to proliferate and persist, forming aggregations and biofilms known as 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]. Often, it is the eventual resurgence of these dormant reservoirs that give rise to the significant percentage of recurrent or relapsing cystitis cases [6].

Indeed, type 1 pili are only one method used by UPEC to invade the host bladder epithelial cells. Other virulence factors which 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 where only a small portion of what exists has been discovered thus far, including various fimbrial and afimbrial adhesins for attachment, siderophores for scavenging essential iron from host cells, as well as 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 [12]. Despite it being the predominant cause of Gram-positive UTIs, relatively little is known regarding the mechanism it uses to invade the urinary tract system or how host cells respond to infection [12]. Some discovered virulence factors of *S. saprophyticus* include extracellular slime, lipoteichoic acids which aid in the adhesion to uroepithelial cells, Aas, an adhesive and autolytic protein which allows for attachment to uroepithelial cells, the adherence factor and haemagglutinin UafA, the collagen- and fibronectin-binding protein SdrI, surface-associated lipase Ssp, serine-rich adhesin UafB, and the enzyme urease [13]. More 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 [12]. Additionally, the authors found virulence factors SssP and SdrI to be important for the persistence but not initial colonization of the bacterium [12]. 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 [14].

Although less studied than the UPEC, findings associated with the pathogenesis of *S. saprophyticus* thus far suggests that similar to UPEC, an array of virulence factors are involved such that no single factor is sufficient to cause disease. Rather, it is the timely, procedural expression of multiple, potentially redundant factors interacting together that contributes to the successful establishment of cystitis [13].

Treatment and Preventive Measures

Current treatment for cystitis involves the use of antibiotics [3]. However, the major drawback to this type of treatment is the development of antibiotic resistance by uropathogens [2]. As such, a wide variation in prescribing practices currently exists [15].

With treatment options being limited and increasing development of antibiotic resistance by uropathogens, it is important to take preventive measures against the development of cystitis. One type of such prevention is the consumption of cranberry juice, which has been widely used for several decades for the prevention and treatment of UTIs [2]. It has been suggested that cranberry reduces the development of UTIs by preventing bacterial adherence to uroepithelial cells [2, 16]. Specifically, cranberry inhibits the binding of P-fimbriae of UPEC via mannose-specific, lectin-like structures to mannose-like residues on mucosal cells [4]. It was supported by past studies where the interaction of *E. coli* with uroepithelial cells was shown to be mediated by a receptor containing D-mannose; both D-mannose and methyl γ-D-mannopyranoside inhibited this adherence in a dose-dependent manner, displacing the uropathogens from their attachment sites on epithelial cells [4]. However, the exact mechanism by which cranberry juice works to prevent UTIs remains to be elucidated.

Other preventive measures, particularly against recurrence, include using long-term, low-dose prophylactic antimicrobial taken at bedtime [10]. However, such applications are susceptible to the development of resistance and may lead to health problems.

If cystitis does not get treated properly, more complicated infections may arise, particularly if the uropathogens ascends to the kidneys and cause infection, a condition known as **pyelonephritis** [2].

Pyelonephritis

Pyelonephritis is the inflammation of the upper urinary tract system commonly caused by bacterial infection. Women are more likely than men to develop pyelonephritis due to a shorter urethra. Other factors that predispose people to pyelonephritis are diabetes, kidney stones, bladder tumours, 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 [17, 18]. Gram-positive bacteria such *as Staphylococcus saprophyticus, Enterroccus faecalis, Streptococcus galactiae* as well as *Mycobacterium tuberculosis* are also implicated in rare cases of pyelonephritis but are rarely described in literature [17, 18]. Research in past years has focused on two common ways for bacteria to infect the kidneys and upper urinary tract (UT); the ascending mechanism and the hematogenous mechanism [18]. 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 then 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 flow of urine may also contribute to the retention of con-

taminated urine in the bladder that may propagate into the upper urinary tract and 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 [18]. 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 papers 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 [19–21]. While much research has gone into understanding these virulence mechanisms what remains uncertain is, however, whether any single virulence factor is responsible for pathogenesis. In the case of ascending mechanism, the bacteria originate from the host's own fecal sources [17, 18]. Upon introduction into the urethra, the bacteria migrate upwards towards the bladder via mechanisms including the use of flagella, a whip like structure that propels bacteria forward [19]. As the pathogens migrate up the lower urinary tract they interact with and attach to uroepithelial cells using specially expressed structures on their surface known as adhesins, mainly P fimbriae and Type 1 fimbriae [19–24]. Adhesion to the uroepithelium allows them to in part overcome an important host protective mechanism in urine flow.

In addition to overcoming urine flow, uropathogens also have to evade the immune system. Once bacteria ascend the ureter, they adhere to renal tubular epithelial cells. This adhesion, along with Lipopolysaccharide (LPS) LPS- TLR4 interaction and the disruption of blood cells by haemolysins, trigger signalling pathways which include the ceramide signalling pathway and LPS-induced TLR-4 dependent signalling pathways [22, 23, 25, 26]. This leads to an upregulation of pro-inflammatory cytokines (mainly IL-6 and IL-8) and chemokines (including CC-chemokines MCP-1 and RANTES) which recruit other host immune cells [25, 26].

To overcome this challenge, bacteria express capsular polysaccharide on their surface, which forms a thick protective layer that prevents opsonisation by activated complement components as well as phagocytosis by macrophages and other relevant immune cells [19–21]. While bacterial infection is the actual cause of pyelonephritis, the resultant tissue damage is not necessarily caused by the bacteria themselves, but rather the immune response they activate; particularly granulocytes such as polymorphonuclear leukocytes (PMNLs) which cause degenerative changes to renal tubular epithelia such as mitochondrial swelling, dilated endoplasmic reticula, increased electron lucency of the cytoplasm and formation of cytoplasmic vacuoles [27]. Conversely, depletion of PMNLs in a rat model almost completely abrogates renal parenchymal damage with minimal bacterial invasion for up to 40 h [27].

Since free iron is limited in the urinary tract, some bacteria express haemolysin, which is an enzyme that ruptures red blood cells thereby forcing the release of iron into

the urinary environment where they capture it using siderophores such as Enterobactin, Yersiniabactin and Aerobactin, which are iron scavenging proteins [19–21].

Infection with *Proteus mirabilis* and some strains of *E. coli* is further complicated by the fact that they express urease, an enzyme that breaks down urea in the urine as an energy source to produce ammonia, resulting in a significant rise in urine pH [28]. In addition to promoting ammonia-induced cytotoxicity of the renal epithelium, the increase in pH also triggers the precipitation of magnesium ammonium phosphate and the eventual formation of struvite stones, which grow rapidly in some cases forming staghorn stones which are branched stones that take over the majority of the kidneys collecting system [28].

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 any particular condition. In one of a few rare comparison studies, it has been observed that Pyelonephritis-associated E. coli strains often carry 2–3 copies of the pap gene cluster while Cystitis-associated *E. coli* strains only carry one cluster [29]. Similarly, it has also been shown that pyelonephritis and prostatitis *E. coli* isolates exhibit more virulence factors overall than cystitis isolates [29]. 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) [29].

Other observations include different adhesion and growth rates between *E. coli* isolates from pyelonephritis and cystitis. As expected, pyelonephritis strains adhere better to uroepithelial cells, are more likely to mediate mannose-resistant hemagglutination, and are often more P fimbriated due to the increased copy number of pap gene clusters per bacteria [30].

As for growth rates, pyelonephritis *E. coli* strains infect initially at lower concentrations but tend to persist in the bladder, kidney, and urine, so that by the end of a 7-day observation period they are present in higher concentrations in the kidney than are the cystitis strains [30]. In contrast, Cystitis strains colonize the bladder in higher numbers at an early stage (up to 3 days), induce more pronounced histologic changes in the bladder, and are more rapidly eliminated from the urinary tract than pyelonephritis strains [30].

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

Urethritis

Urethritis is a urinary tract condition characterized by the inflammation of the urethra. In adults, urethritis is mainly of infectious nature and usually transmitted by sexual contact [31]. In fact, one of the most prevalent types of sexually transmitted infections (STI) in men is nongonococcal urethritis (NGU) [32]. Pathogenic microbes most commonly responsible for NGU include: *Chlamydia trachomatis* (30–50 % of NGU cases) and *Mycoplasma genitalium* (10–30 % of NGU cases) [32]. Other pathogens found to be implicated in urethritis include *Ureaplasma urealyticum, Haemophilus* species, *Streptococcus* species, *Gardnerella vaginalis,* herpes simplex viruses, adenoviruses and *Trichomonas* species [32, 33]. Another form of urethritis is gonococcal urethritis (GU). GU is caused by *Neisseria gonorrhoeae* [33]. 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 [34].

C. trichomatis exists in two morphological forms: the intracellular reticulate body form (RB) and the (extracellular) elementary body form (EB) [35]. The EB form of *C. trichomatis* is metabolically inactive, but is infectious [36, 37]. It is this form of *C. trichomatis* that is responsible for the initial colonization of the urethra and in turn the development of NGU. When the EB form of *C. trichomatis* enters the urethra, they infect susceptible host cells by using a heparin sulfate-like glycosaminoglycan molecule on their cell surface to bind an unknown host cell receptor [37]. Although the receptor is unknown, it is known that these host receptors are localized to the apical surface of polarized cells thus, making the genital epithelium the target of *C. trichomatis* [36]. 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 actin recruitment and promotes internalization [36, 38]. When the EBs are endocytosed, they are placed into a membrane bound compartment known as an inclusion and is further transported into the perinuclear location in the infected cell. Following internalization, in approximately 6–8 h, the *C. trichomatis* EBs differentiate into RBs [37]. RBs are metabolically active and are mainly responsible for *C. trichomatis* proliferation via binary fission [35, 37]. In 24–72 h, the newly generated *C. trichomatis* progeny differentiate into EBs and induce cell lysis in order to escape and infect more cells [36, 37]. In response to *C. trichomatis* infection, the host initiates a proinflammatory Th1 immune response [39] inducing cell mediated immunity resulting in an inflamed urethra. In the case of an extreme Th1 immune response, tissue damage may result [40].

For *M. genitalium* to cause NGU, colonization of the urogenital tract is imperative. When *M. genitalium* are 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 [41]. 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. trichomatis*, *M. genitalium* is found to localize to the perinuclear space and replicates by binary fission [42, 43]. 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 acids and provides the *Mycoplasma* with a source of nucleotide precursors for growth and pathogenesis [43]. A previous study (using lung fibroblast cells) has shown that at 96 h post infection, *M. genitalium* lysed the infected cell and released progeny into the surrounding environment [44]. The immune response produced by the host towards *M. genitalium* is largely dominated by polymorphonuclear leukocytes [43]. Lipoproteins found on the cell surface of *M. genitalium* also contributes to the inflammatory immune response. When immune cells interact with these lipoproteins, proinflammatory cytokines are produced and in some cases, this interaction may lead to necrosis or apoptosis [45].

In the case of GU, *N. gonorrhoeae* is responsible for the infection of urogenital epithelial cells [46]. When *N. gonorrhoeae* encounters urogenital epithelial cells, the bacteria rely on Type IV Pili to adhere to these cells [47]. More specifically, for adherence, the Type IV Pili interact with a human-specific complement regulatory protein 46 (CD46) [47]. Upon adhesion, outer membrane bacterial proteins known as opacity protein adhesin (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 [46–48]. 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 [46, 49]. Following internalization by host urogenital epithelial cells, the gonococcus undergoes transcytosis to reach the basilar side [50]. The intracellular processes of *N. gonorrhoeae* still remain unclear [46]. When *N. gonorrhoeae* infection is detected by the immune system, a strong proinflammatory response is elicited [51]. To further contribute to inflammation, *N. gonorrhoeae* express lipooligosaccharide (LOS). Urogential epithelial cell interaction with bacterial LOS has been shown to result in the production of cytokines and chemokine such as TNF-α, IL-1β, IL-6 and IL-8. As a consequence of cytokine and chemokine secretion, polymorphonuclear leukocytes are recruited and lead to inflammation [46].

Bacterial Prostatitis

Inflammation of the prostate gland is known as prostatitis. This condition affects approximately 9 % of Canadian men per year [52]. Furthermore, in the United States, between 1990 and 1994, there were over two million hospital visits for prostatitis per annum [53]. Prostatitis can be further differentiated into four categories: acute bacterial prostatitis, chronic bacterial prostatitis, abacterial prostatitis and

prostatodynia. In all the cases of prostatitis, bacterial prostatitis only accounts for 5–10 % of the cases [54]. In acute bacterial prostatitis, the major causative pathogen is *Escherichia coli* and other bacteria involved are *Pseudomonas*, *Klebsiella* and *Enterococcus* species [55]. Similarly, in chronic bacterial prostatitis, *E. coli, Klebsiella* species, and other Gram-negative bacteria including *Proteus* species are responsible for the inflammation of the prostate [53]. This section will focus of the pathogenesis of the *E. coli* and *Klebsiella* species which are common in both acute and chronic bacterial prostatitis.

In acute bacterial prostatitis, uropathogens reach the prostate by ascending the urethra, ascending urethral catheters or can be due to sexual transmission [53]. In the case of *E. coli* different strains have been found in prostatitis patients and each strain may possess similar and unique virulence factors. One important virulence factor of *E. coli* are P-fimbriae. A study conducted by Andreu et al. has identified P-fimbriae in 53 % of patients in their prostatitis group [56]. Fimbriae-associated adhesins in general help mediate attachment to host cells by binding to glycoconjugate receptors [57]. Bacteria possessing P-fimbriae may also have better localization to the prostate as the presence of bacteria with these fimbriae have been associated with increased tropism for the prostate [58]. Furthermore, the presence of P-fimbriae has been shown to enhance urinary tract colonization [57]. Some *E. coli* strains found in patients with prostatitis also possess hemolysin [56]. Hemolysins produced by *E. coli* serve various functions which include: cytolytic activity to damage tissues, help in iron acquisition, protection of *E. coli* by lysing phagocytic cells, aid bacterial invasion, and contribute to a persistent infection within glandular prostate tissue [56]. Many of the uropathogenic *E. coli* isolated from prostatitis patients have also been shown to produce cytotoxic necrotizing factor type 1 (CNF-1) [56, 59]. CNF-1 has been shown to increase the ability of polymorphonuclear leukocytes to generate superoxide species and to adhere to epithelial T84 monolayers. Conversely, CNF-1 decreases the phagocytic function of these leukocytes [60]. CNF-1 may also increase inflammation *in vivo* as rat prostates infected with CNF-1^+ *E. coli* exhibited increased and more severe inflammation compared to prostates infected with CNF-1^- *E. coli* [61]. If the *E. coli* strains possess the ability of forming biofilms, acute bacterial prostatitis patients infected with these biofilm forming *E. coli* may develop chronic bacterial prostatitis. Biofilm formation in the prostate can allow *E. coli* to persist and in turn, recurrent urinary tract infections characteristic of chronic bacterial prostatitis will result [62].

For *Klebsiella* species to initiate infection and colonization, their attachment to urogenital epithelia is mediated by Type 1 Pili and Type 3 Pili [20]. Type 1 Pili bind to mannose-containing structure on host cells, while the receptor to which Type 3 Pili bind is still unknown [63]. While in the host, *Klebsiella* species also produce capsular antigens, which forms a protective cover around the bacteria. These capsules are made from acidic polysaccharides and serve to help *Klebsiella* evade the immune system, by preventing phagocytosis by polymorphonuclear granulocytes and confers protection from bactericidal serum factors. Furthermore, *Klebsiella* expresses enterocholin, a high affinity iron scavenging protein (siderophore), which acts in iron sequestration from the surrounding environment [20, 64]. Another virulence factor

that *Klebsiella* species possess is lipopolysaccharide (LPS) [20]. LPS may contribute to inflammation as LPS is implicated in TLR-4 signaling [65]. Activation of TLR-4 has been shown to be associated with the production of IL-17, a pro-inflammatory cytokine [66]. Similar to *E. coli*, *Klebsiella* are capable forming biofilms which render infections more difficult to eliminate. These biofilms provide protection from antibiotics and from the host immune system [67]. As a result of biofilms' protective ability, *Klebsiella* species may be able to cause chronic bacterial prostatitis.

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Chapter 3
Pathogenic Mechanisms of Uropathogens

Ryan Chanyi, Jeremy P. Burton, and Peter A. Cadieux

Abstract Urinary tract infections (UTIs) can affect both men and women at almost any stage in their lifetime. While the vast majority of these are not life-threatening, they cause significant morbidity to patients and place a heavy burden on healthcare systems worldwide. This is further complicated by the use of urinary drainage devices such as catheters and stents, which provide additional sites for bacterial/fungal attachment and biofilm development. Despite being exposed to a wide array of antagonistic environmental conditions and the host immune system, uropathogens are generally very successful at establishing infection. This is mainly due to the plethora of pathogenic mechanisms they utilize that provide an advantage over the host. In this brief review, we discuss a small subset of the mechanisms used by uropathogens including the appendages, proteins and sugars used to adhere to surfaces, the invasion into host tissues, immune evasion strategies and antibiotic resistance. This work illustrates the complexity of the interaction between the urinary tract and uropathogens, and supports the development and application of multi-faceted strategies for infection prevention and treatment.

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Introduction

Urinary tract infections (UTIs) are one of the most common diseases caused by bacteria, representing the most abundant cause of hospital acquired infections [1]. Roughly 50 % of all women will experience a UTI within their lifetime with 44 % of those experiencing recurrence [2]. A single mechanism to establish an active or recurrent UTI does not exist. Bacteria employ a plethora of mechanisms, often simultaneously, to assist in many key steps to survive in the host [3, 4]. In this chapter only a few select mechanisms can be discussed including adherence to the host epithelium, host immune modulation, nutrient acquisition, host-cell invasion, biofilm formation and antibiotic resistance. Although many different bacteria can cause UTI's, 80 % have been associated with *Escherichia coli* and as such, this species has been a major focus of study. Therefore, although other uropathogens will be discussed periodically, uropathogenic *E. coli* (UPEC) will be the primary focus.

Adherence to Epithelial Cells; Type I Fimbriae and P Fimbriae

An important step in infection establishment at almost any anatomical site is host cell adherence. For uropathogens, numerous mechanisms assist urothelial attachment, critical for avoiding clearance during micturition (urination). Some UPEC strains produce several long (~1 μm) extracellular fimbriae with adhesive tip proteins used to bind specific receptors on the mucosal surface [5]. In a model of cystitis, Type I fimbriae with the FimH adhesin bind to terminal mannose moieties on Uroplakin (UP) Ia found on umbrella cells of the urothelium ([6–13]). This binding induces numerous physiological changes in the host cell including an increase in intracellular calcium, the phosphorylation of a UP signalling complex and Rho-GTPase activation, leading to local rearrangement of the actin cytoskeleton and UPEC engulfment via a zippering mechanism [11, 14–18]. At the same time, bacterial attachment via FimH triggers host exfoliation of the terminally differentiated superficial umbrella cells in an effort to remove those infected [11, 19]; however, while this can help clear a large number of the invaders, it also exposes the underlying undifferentiated cells to attack. Further supporting the critical nature of Type I fimbriae in UTI development, FimH residues that enhance virulence have been shown to be selected for in UPEC strains [20], the fimbrial regulator *fimX* can be used as a molecular marker for identifying UPEC strains [21] and *Klebsiella pneumonia*, the second-most prevalent Gram-negative UTI pathogen, uses a virtually identical system during infection [22]. Once inside the host cell, the bacteria are sheltered from many extracellular host-defense mechanisms and can establish an active infection or a reservoir for recurrence.

Another fimbrial system associated with UTI is the P fimbrial system, strongly linked to ascending UTI and pyelonephritic infections [23–25]. While generally similar in structure to Type I fimbriae, their tip adhesin PapG binds specifically to

glycosphingolipids (Gal-(α1-4)-Gal) found on renal epithelial cells. PapG adhesion signals host release of ceramide, a Toll-like receptor 4 agonist which leads to local inflammation and pain. Although P fimbriae are not essential for lower UTI development, a synergy between Type I and P fimbriae has been shown to enhance the rate at which UPEC are able to invade renal epithelial cells and thus establish more serious infections [26]. This is a similar observation to the mannose-resistant *Proteus*-like (MR/P) fimbriae found on some uropathogenic strains of *Proteus mirabilis*; a cause of serious UTI's and acute pyelonephritis [27]. The MR/P fimbriae are not essential for infection but do contribute to virulence by eliciting a strong immune response *in vivo*. Akin to P fimbriae in UPEC, the role of MR/P fimbriae in urothelial adherence is not entirely determined. Strains lacking MR/P fimbriae are still able to adhere to urothelial cells after 1 h, albeit not as well as strains with MR/P fimbriae [27].

Although less studied, additional fimbriae have been implicated in UTIs. Both S and F1C fimbriae can bind epithelial and endothelial cells of the lower urinary tract and kidney [28–30]. S fimbriae have even been associated with *E. coli* strains that cause sepsis, meningitis and ascending UTIs [31]. Due to the implication of numerous fimbrial systems as strong virulence factors, a lot of research has gone into using inhibitors of fimbrial production and assembly as targets to increase sensitivity to many antibiotics that are no longer effective [32, 33].

Biofilm Formation on Implanted Devices

In addition to fimbriae, many uropathogens also produce adhesins expressed directly on the bacterial surface. These adhesins are not only used to attach to host cells but also to implanted devices such as urinary catheters and stents. When bacteria attach to devices they typically form biofilms, polymicrobial communities of bacteria enclosed within secreted extracellular polymeric substances (EPS) [34]. Due to biofilm structure and the reduced growth rates that typically occur within them, these communities are generally more resistant to harsh environmental conditions such as urine flow, pH changes, host immunity and antibiotic exposure. Furthermore, bacteria within biofilms become chronically exposed to sub-inhibitory concentrations of any clinically-administered antibiotic, inducing drug resistance, biofilm enhancement and the production of dormant persister cells; all key factors leading to chronic and recurrent infections [35], [36]). Several key adhesins linked to device adherence and biofilm formation are discussed herein.

Antigen 43 (Agn 43) is a self-binding, biofilm-promoting surface adhesin found on many UPEC strains [37]. This autoaggregating protein is so good at forming biofilms that its expression on the surface of non-biofilm-forming *Pseudomonas fluorescens* induced biofilm development similar to that of *E. coli* [38]. Glycosylated Agn 43 also binds to collagen and laminin, host proteins found within the urinary conditioning film that typically deposits on indwelling devices following placement [39].

As mentioned, host proteins and numerous other urinary constituents bind to the surface of any foreign device placed in the urinary tract within minutes. Many bacterial surface proteins (fimbrial and afimbrial) are subsequently able to utilize these factors for device attachment and biofilm formation. Examples include a number of fibrinogen-binding proteins such as Clumping factors A and B (ClfA and ClfB) and Iron-regulated surface determinant A (IsdA) on *Staphylococus aureus* [40, 41] and Fss1, Fss2, Fss3 and the Endocarditis and biofilm-associated pilus adhesion (EbpA) on *Enterococcus faecalis* [42]. Interestingly, EbpA has also been shown to directly promote biofilm formation on catheters and vaccination with the protein prevented biofilm development and catheter-associated UTI in a murine model [43]. Furthermore, Iron-regulated surface determinant A (IsdA) recognizes cytokeratins [40, 44] and heme [40] in addition to fibrinogen (all three compounds have been recovered from indwelling urinary devices), demonstrating the versatility of many of these bacterial proteins. Collectively, this data highlights how factors within the urinary conditioning film not only mask the exposed surface of any urinary device placed in a patient but also offer a plethora of microbial adherence options. These surface interactions represent a crucial step in the formation of indwelling device associated biofilms, and thus, is a significant area of study. Once formed, biofilms are extremely difficult to remove and typically require device removal alongside antimicrobial therapy to completely eradicate the infections.

Intracellular Biofilm Formation; Long Filament Formation; Quiescent Intracellular Reservoirs

As described previously, Type I fimbriae induce invasion into urothelial cells by inducing cytoskeletal rearrangement via binding to UPIa and UPIIIa. The natural response by the host is to shed the superficial umbrella layer; however, many UPEC strains are able to penetrate deeper into the underlying undifferentiated bladder cells to avoid clearance [45]. Upon engulfment into bladder epithelial cells, bacteria are generally enclosed within an acidic vacuole where they are unable to replicate efficiently [46]. Following this, the pathogen can then be expelled from the cell [47] or can escape from the vacuole into the cytosol. If escape occurs, the UPEC will begin reproducing quickly to form what is termed an intracellular biofilm community (IBC). Similar to extracellular biofilm formation, the cells are tightly packed, surrounded by EPS and contain subpopulations that undergo differential gene expression [48–50]. Numerous factors control this process including Type I fimbriae [51], capsular polysaccharide [52, 53] and five additional Type I-independent mediators whose functions are currently unknown [54]. IBC growth ultimately becomes limited based upon host cell size, triggering UPEC to alter its cellular morphology to a long filamentous cell type. These filaments are able to flux out of the cell to reach neighboring bladder epithelial cells and establish new IBCs. Naturally the host will try to exfoliate the superficial bladder epithelium to clear the infected cells. The invasion by UPEC of the underlying cells leads to the development of quiescent intracellular reservoirs (QIR).

Bacteria within the QIR are dormant, non-replicating cells that are highly resistant to antibiotics, and as such, are able to persist [55–57]. Occasionally a subset of these persister cells will become active and cause a new UTI [55].

Hemolysin and Cytotoxic Necrotizing Factor

In the bladder, exfoliation of the superficial umbrella cells can be beneficial or detrimental to the host. In one aspect, physical removal of infected cells is a quick and easy way to clear many of the bacteria in an infection. On the other side, it exposes undifferentiated cells below that, if infected, are more difficult to treat. α-Hemolysin (HlyA) is a pore-forming toxin produced by UPEC that is able to lyse many cell types including red blood cells, natural killer cells [58] and bladder epithelial cells (BECs). At subcytotoxic concentrations, HlyA activates serine proteases within BECs which lead to the degradation of a cytoskeletal scaffolding protein, paxillin [59]. This promotes the exfoliation of the bladder epithelium. HlyA also induces dephosphorylation of Akt which modulates signalling cascades, including a reduction in the NF-κB response [60]. This activation of proteases is not only limited to the bladder epithelium but has also been shown in macrophages, inhibiting proper function [59]. Cytotoxic necrotizing factor 1 (Cnf1) is another toxin secreted by UPEC via outer membrane vesicles [61, 62] that is able to induce bladder inflammation and submucosal edema in a murine UTI model [63, 64]. Cnf1 constitutively activates RhoA, Rac1 and Cdc42 which results in the formation of actin stress fibers, filopodia, lemellipodia and eventually apoptosis [65–67]. Overall, it is apparent UPEC isolates are able to use secreted toxins to promote exfoliation of host cells, establish an active infection and modulate the host immune response.

Siderophores

Iron is an essential cofactor required for many processes including electron transport and nucleotide biosynthesis. As the bioavailability of free iron in the host is limited, it is critical that bacteria develop methods for sequestering it to survive. The majority of iron in the host is bound to heme or heme-containing proteins such as hemoglobin and hemopexin. UPEC strains produce four different siderophores that sequester iron from these host proteins and transport it back into the bacterial cell [68]. Enterobactin is the strongest known siderophore produced by Gram-negative bacteria. Because of its strong binding, enterobactin practically "rips" the iron from heme and translocates it across the bacterial cell wall in a TonB-dependent manner [69]. Upon entry the iron is reduced, thereby decreasing its binding affinity for enterobactin and allowing its release and utilization. Lipocalin-2 is a secreted host-protein that binds bacterial enterobactin, essentially blocking its iron sequestration function and thus severely limiting bacterial growth and survival. While enterobactin is fairly widespread among

Gram-negative bacteria, other siderophores such as salmochelin, aerobactin and yersiniabactin are far more prevalent in pathogens. Salmochelins are a group of glucosylated forms of enterobactin that lipocalin-2 is unable to bind, thus allowing them to remain unaffected by host defenses [70]. Overall, the addition of glucosyl groups by the IroB glucosyltransferase and further modifications by other *iro* gene cluster members can result in the production of 9 structurally different salmochelins (3 cyclic and 6 linear), showing their diversity and importance in bacterial survival. Furthermore, the expression of the salmochelin receptor IroN has been shown to be upregulated in IBCs. Aerobactin is a siderophore strongly associated with UPEC that is generated through the oxidation of lysine. Unlike enterobactin, aerobactin delivers iron directly to iron-requiring sites within the bacterial cell and can be recycled without hydrolysis for immediate reuse. As its name implies, Yersiniabactin is a phenolate siderophore required for virulence in *Yersinia pestis* [71]. However, it is also strongly linked to UPEC isolates where it is associated with UTI establishment [72] as well as the development of more serious upper UTIs and pyelonephritis. Recent studies have shown that yersiniabactin can also bind copper [73] and vaccination with its receptor can prevent infection in animal models [74].

Urease

Urease production has been highly studied in the gastric ulcer-producing bacterium, *Helicobacter pylori*. Uropathogens such as *P. mirabilis, Klebsiella* species and some *E. coli* also produce urease [75], which has been linked to struvite stone formation and pyelonephritis. Urease hydrolyzes urea in the urine into ammonia and carbonate. This significantly increases the local pH resulting in the precipitation of ions that are normally soluble in urine. Magnesium ammonium phosphate (struvite) urinary stones can lead to calculus formation within the renal pelvis and result in the blockage of urine flow through catheters. Bacteria found within these stones are also more protected from the host immune response and antibiotic treatment leading to resistance and recurrence.

Antibiotic Resistance

There are many factors which contribute to antibiotic resistance or tolerance, a couple of which have already been mentioned such as metabolic dormancy and avoidance. Resistance is also acquired through additional strategies including antibiotic inactivation, efflux from the cell and target modification [76]. These strategies are typically acquired via random genetic mutation and/or novel gene acquisition. Examples of mutations include those that alter the active site of a critical bacterial enzyme where antibiotic binding typically occurs, such that it is no longer able to bind, as well as those that alter or upregulate bacterial efflux systems to promote or enhance antibiotic removal from the bacterial cell. Novel gene acquisition includes those encoding β-lactamase enzymes or antibiotic resistant transpeptidases. These novel genetic

elements can be picked up through natural transformation or transferred from other bacteria via mobile genetic elements (e.g., transposons and plasmids). For example, genes for β-lactamases like CTX-M-27 are commonly transferred between bacteria on plasmids. These enzymes degrade a large number of β-lactam antibiotics including cefotaxime, ceftazimide and aztreonam. CTX-M-27 originated in UPEC strains in Asia and can now be found spreading into the Czech Republic [77] showcasing the remarkable ability for these pathogens to transfer resistance.

Another example of resistance by UPEC involves the aminoglycosides. Many strains encode aminoglycoside-modifying enzymes that alter the active site such that it is unable to bind the bacterial ribosome and block protein synthesis. For example, aac(3)-IIa acetylates an amino group on the aminoglycoside while ant(2″)-la adenylates a hydroxyl group [78]. Both modify the antibiotic so it can no longer bind to the 30S ribosome, conferring resistance to multiple clinically-relevant aminoglycosides. These enzymes are of growing concern as Soleimani et al. [79] showed that nearly 40 % of UPEC strains isolated from urine samples in an Iranian hospital were resistant to gentamycin, kanamycin and tobramycin. To avoid or overcome these challenges, aminoglycosides and β-lactams are often prescribed together. These two classes have been shown to work well synergistically, attacking both bacterial peptidoglycan and protein synthesis simultaneously, in addition to decreasing resistance potential.

Trimethoprim and sulfamethoxazole are folic acid (folate) synthesis inhibitors that have been used in combination for decades in the treatment of UTI, as they effectively target different stages of the process. They are also both generally well tolerated and cost effective. However, resistance is increasing with one study demonstrating 86 % of UPEC isolates from patients resistant to the combination [80]. While random point mutations in the target genes have been shown to confer this resistance, it is most commonly acquired through plasmids encoding modified homologues of the genes that are functional but fail to bind the antimicrobials [81].

Briefly discussed above are the populations of bacteria within device biofilms and IBCs that develop into dormant persister cells. These subpopulations alter their metabolism such that many antibiotics that rely on active bacterial growth and replication for efficacy become ineffective. While these persister cells form naturally at low levels during general bacterial growth, under stressful growth/survival conditions their generation is greatly upregulated. It is important to note that these organisms are not antimicrobial resistant but instead are termed tolerant, waiting until the antibiotic treatment has subsided to resume metabolic activity and re-establish an active infection [82]. This phenomenon is a major driving force behind recurrence as there is currently no method to target and kill all persister cells.

Host Immune Modulation

In order to establish an infection, bacteria must be able to survive the host immune response. Many UPEC strains encode multiple mechanisms that modify the local environment to aid in this goal. A recent study demonstrated that several UPEC strains were able to significantly increase the secretion of anti-inflammatory

cytokines such as IL-5, IL-10 and IL-17 in a mouse model of infectious epididymitis [83]. In addition, UPEC strains have shown the ability to suppress the NF-kappaB pathway in urothelial cells, decreasing the secretion of several pro-inflammatory cytokines [84]. Both paradigms result in the suppression of a Th-1 mediated host immune response, resulting in a more suitable environment for UPEC to establish an infection. Finally, some UPEC strains have been shown to possess a variant of the periplasmic protein YbcL that when expressed inhibits the transepithelial migration of neutrophils to the site of infection [85].

It is important to note that only a small fraction of the pathogenic mechanisms of uropathogens have been discussed herein. Furthermore, it should be noted that many uropathogens will use multiple mechanisms simultaneously. Therefore, the success of a pathogen will rarely rely on a single trait but instead on a combination working synergistically. Generally speaking, pathogenic microorganisms are simply geared toward population survival and will exploit any weakness possible in the host's armour.

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Chapter 4
Urosepsis-Pathogenesis and Treatment

Samir Bidnur and Ryan K. Flannigan

Abstract Urosepsis is a life-threatening infection that results from the interaction of bacteria and bacterial products with the host immune system, resulting in a clinically unstable patient. Pathogen factors include the virulence of particular bacterial strains/subtypes which produce toxins, most commonly components of the bacterial cell wall, that amplify and exaggerate the host immune response. Patient factors that contribute to the development of urosepsis include a compromised immune system incapable of effectively clearing infection, as seen in patients with uncontrolled HIV or patients taking chronic immunosuppression (i.e. chronic steroid use). Certain urologic conditions, specifically the obstruction of urinary flow secondary to urolithiasis or benign prostatic hyperplasia (BPH), can result in the rapid development of urosepsis. Successful management of sepsis requires early identification of clinical sepsis, prompt fluid resuscitation and administration of antibiotics, and relief of obstruction.

Clinical Introduction

Urosepsis refers to a severe infection (−sepsis) from a genitourinary source (Uro-) [1]. These infections start with a focal source, in this case from the genitourinary system; however, products of the infection and host immune response lead to a systemic inflammatory response with associated downstream physiologic and clinical changes. The severity of sepsis from least to most severe respectively, are classified as:

1. Sepsis
2. Severe Sepsis
3. Septic Shock

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Distinguishing among these severities are 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	≥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 – Criteria 1 + ≥ 2 Criteria 2
2. Severe Sepsis – Criteria 1 + ≥2 Criteria 2 + ≥1 Criteria 3
3. Septic Shock – Criteria 1 + ≥2 Criteria 2 + refractory hypotension ≤90 mmHg

The associated mortality varies with number of criteria involved. Sepsis mortality varies between 7 and 17 % if 2 and 4 criteria 2 are satisfied respectively; severe sepsis mortality increases by 15–20 % for each organ system involved from criteria 3; mortality in septic shock is between 50 and 80 % [1].

Sources and Pathogenesis of Urosepsis

The genitourinary tract accounts for 20–30 % of sepsis [2]. 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 % [3]. 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: E. coli, Proteus spp., Klebsiella spp., Enterobacter, P. aeruginosa, and Staphylococcus aureus [1, 4]. In immunocompromised patients Candida spp. and Pseudomonas spp. should be considered [5].

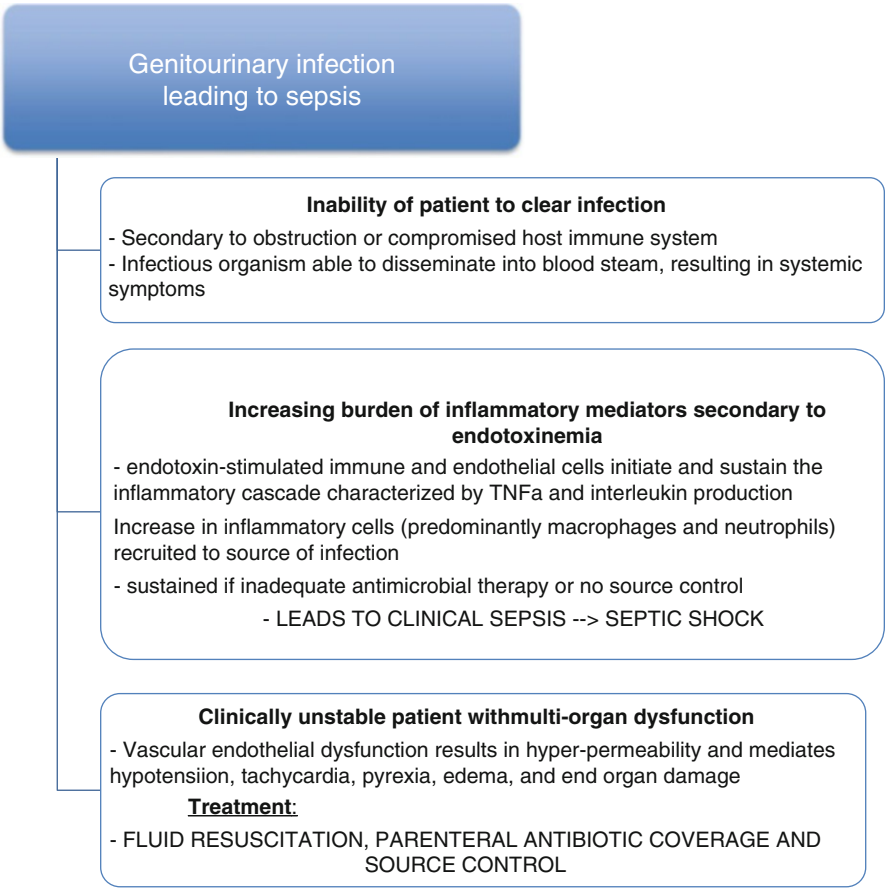
Bacterial endotoxins, such as lipopolysaccharide (LPS) from the cell wall of gram-negative organisms appear to mediate the systemic manifestation of sepsis. These bacterial components activate the inflammatory, coagulation, and complement systems, stimulating the activity of monocytes, macrophages, neutrophils, and dendritic cells, amongst other inflammatory cell subtypes [6]. LPS-stimulated monocytes play a central role in mediating clinical sepsis, and produce tumour necrosis factor alpha (TNFa) and interleukin (IL)-1 at LPS concentrations of 25–50 pg/mL [7]. In addition to stimulation of inflammatory cells, endotoxin also directly binds receptors in the endothelial cell membrane, which also promotes pro-inflammatory mediators [8].

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 TNFa, IL-6 and neutrophil response, occurred earlier and was more severe in the bolus group compared to the infusion group [9]. 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 TNFa has been shown to correlate with death from urosepsis [10, 11] (Table 4.1).

Management

Managing urosepsis requires a prompt recognition, early goal directed resuscitation, broad-spectrum parenteral antibiotics, and source control [12]. Diagnosis is initiated with a focused history with the goal of identifying **criteria 1 above**. Enquiry on history 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 as per **criteria 2 above**. Focused exam should assess flank and abdominal

Table 4.1 The factors that cause a genitourinary infection to progress to sepsis are outlined below



tenderness, and palpation of the scrotum and perineum for crepitus. The latter is of paramount importance for the early detection of Fournier's gangrene.

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 [13] in addition to hydronephrosis and urolithiasis [14].

Early goal directed therapy are required for reducing mortality and optimizing outcomes as described in Rivers Protocol [12]. This involves supporting the patients 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 [12]. Early initiation of empiric parenteral antibiotics, ideally within 1 h of presentation, is essential to minimize mortality [15, 16]. The author recommends searching the patients past medical records for history of a resistant organism. Antibiotic selection should be initially broad to cover bacteria common to urosepsis (see above), consider local patterns of resistance and regional antibiograms, patient allergies, and pharmacokinetics & dynamics of urinary tract involvement and tissue penetration [17]. 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 [18–24]. 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 are necessary [20, 21].

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 the respective clinical scenario.

- Obstructed pyelonephritis
  - Requires urgent retrograde ureteric stent or nephrostomy tube to decompress an infected system [25, 26].
- Emphysematous Pyelonephritis
  - Consider nephrectomy, or percutaneous drainage if not clinically responding to medical management [27–29].
- Renal or Peri-renal Abscess
  - May consider percutaneous drain if ≥3 cm and failure to respond to optimal medical management [30].
- Acute prostatitis
  - If lack of clinical improvement and associated abscess, may consider trans-urethral resection, or trans-rectal drain [31, 32].

- Fourniers Gangrene
  - Requires immediate surgical debridement [33].
  - Can be rapidly progressive, requiring repeat operation to ensure complete debridement

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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Chapter 5
Struvite Stone Formation by Ureolytic Biofilm Infections

Logan N. Schultz, James Connolly, Ellen Lauchnor, Trace A. Hobbs, and Robin Gerlach

Abstract This chapter describes how urinary tract infections can lead to stone formation. The most frequent type of infection stone is struvite ($MgNH_4PO_4 \cdot 6H_2O$), although it is common that struvite stones and infections are associated with other stone types, often forming large staghorn calculi. A complete understanding of struvite stone formation requires knowledge of the pathogen biology, including metabolic activity and motility, as well as a basic understanding of how minerals form.

The pathogens responsible for struvite stones are those that break down urea into ammonium (NH_4^+) and inorganic carbon. This reaction, known as ureolysis, increases the pH of urine and the concentration of NH_4^+, thus increasing the saturation index of struvite. If supersaturation is reached, i.e. the ion activity product (IAP) is greater than the ion activity product at equilibrium (K_{sp}), struvite stone formation is possible.

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An important consideration with urinary tract infections is that pathogens often form attached communities, known as biofilms, which help them to survive physical and chemical stresses. Not only are biofilm-related infections more difficult to treat, but they can facilitate stone formation by creating gradients in chemical concentrations near surfaces. Modern laboratory bioreactors and computer models, described in this chapter, are improving our understanding of how and why infection stones such as struvite form. Current treatment options for infection stones can be painful or ineffective. As more is learned about the complex microbe-fluid-mineral interactions, less-invasive treatments and more-effective prevention strategies will be developed.

The upper urinary tract of a healthy person is generally considered to be sterile, and it has long been known that a **urinary tract infection** (UTI) can lead to stone formation (**urolithiasis**). Around 400 B.C., long before we knew about bacteria, Hippocrates is believed to be the first person to associate the putrification of urine with stone formation [1, 2]. Furthermore, he recognized that drinking fluids increases urine volume and improves the condition of patients. Our understanding of infection stones has since improved considerably, but additional insight appears necessary in order to develop less invasive treatments and more effective prevention strategies. In the modern developed world, approximately 15 % of renal calculi are believed to be caused by infections [3]. Infection stones are more prevalent in women due to a higher infection rate, as well as in developing countries where sanitation is poor [4].

A mechanistic understanding of stone formation resulting from UTI pathogenesis requires a multi-disciplinary approach: Pathogen biology, chemistry, mineralogy and fluid transport processes must all be considered to determine the optimal treatment and prevention strategies. In this chapter we discuss the basic roles of these fundamental topics, with a focus on the most common type of infection stone, magnesium ammonium phosphate hexahydrate, also known as **struvite** ($MgNH_4PO_4 \cdot 6H_2O$).

Urinary Tract Infections

Many bacterial species can cause UTIs, but the species that are most relevant to struvite stone formation are those that produce the enzyme urease to break down urea ($CO(NH_2)_2$), resulting in increased urine pH. The most studied urease producer in the urinary tract is *Proteus mirabilis*, however many others exist [5, 6]. For example, a clinical study by Bichler et al. revealed that approximately 30 % of cultured urinary pathogens were urease producers. Table 5.1 shows the ureolytic species isolated in the study [5].

Urease catalyzes the hydrolysis of urea, a process often called **ureolysis**, producing two molecules of ammonia (NH_3) and one molecule of inorganic carbon (Eq. 5.1). Ammonia is a Brønsted-Lowry base (i.e., a proton acceptor) and therefore generates hydroxide (OH^-) ions that can increase the pH of urine from near neutral

Table 5.1 Frequency of urease producing pathogens, from a clinical study [5]

Organism	Uropathogens cultured	Percent that were urease-positive
Proteus spp.	54	100
Klebsiella spp.	31	84
Staphylococcus spp.	67	55
Escherichia coli	142	1.4
Pseudomonas spp.	20	5
Providencia spp.	1	100
Morganella morganii	1	100
Total	423	28.8

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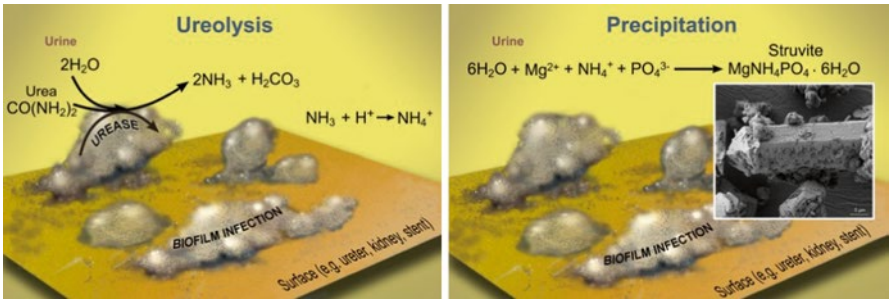

Fig. 5.1 Heterogeneous biofilms can develop on urinary tract surfaces. (*left*) If the biofilm contains microbes that produce urease, urea is hydrolyzed and the chemistry of urine is affected. (*right*) When the chemistry of urine is affected by ureolysis, struvite can precipitate. The *inset* image shows a struvite crystal that developed in a laboratory reactor cultivating a ureolytic biofilm (Trace Hobbs and Ellen Lauchnor, 2014, unpublished data)

to levels as high as 9 [7]. The pH increase and the production of ammonium (NH_4^+), an ionic constituent of struvite, promotes precipitation.

$$\mathrm{CO}\left(\mathrm{NH}_2\right)_2 + 2\mathrm{H}_2\mathrm{O} \rightarrow 2\mathrm{NH}_3 + \mathrm{H}_2\mathrm{CO}_3 \left(\text{Ureolysis}\right) \tag{5.1}$$

Implanted devices such as stents, catheters, pouches, and meshes increase the risk of UTI occurrence, but infections can also develop without a foreign device. It is likely that the migration of pathogens to the kidney is aided by surface-attached microbes, also known as **biofilms**. Biofilms produce extracellular polymeric substances (EPS), mostly polysaccharides, which provide protection against physical and chemical threats to bacteria. The role of biofilms in oral, skin and indwelling medical device infections has been documented for several decades [8], and biofilms play an important role in the colonization of the urinary tract and migration of infectious bacteria into the kidney. Upstream cellular motility, in conjunction with surface-adhering biofilms, likely allows pathogens to overcome peristaltic flow through the ureters and ultimately infect the kidneys [9] (Fig. 5.1).

In addition to protecting the bacteria, biofilms can play a role in struvite stone formation. At the interface of the biofilm and urine, cells are in direct contact with the substrates required for struvite formation (i.e., urea, Mg^{2+}, PO_4^{3-}), thus there is a close relationship between the ureolytic activity in the biofilm and stone formation. Furthermore, EPS can serve as a matrix glue to allow smaller crystals – not only struvite but also other stones such as calcium oxalate – to combine into larger stones [10].

For several reasons, UTIs can also promote the formation of metabolic stones, i.e. those that are not normally associated with infection [11]. For example, an increase in urine pH promotes the formation of calcium phosphate stones because it shifts the phosphate speciation from HPO_4^{2-} to PO_4^{3-}. Another example is that higher urine pH accelerates the breakdown of ascorbic acid into oxalate, thus promoting calcium oxalate stones [12].

Ureolytic Precipitation of Struvite

The precipitation of struvite ($MgNH_4PO_4 \cdot 6H_2O$), described in Eq. 5.2, requires dissolved ions of magnesium (Mg^{2+}), ammonium (NH_4^+) and phosphate (PO_4^{3-}). Sterile urine contains dissolved Mg^{2+} and PO_4^{3-}, and sufficient concentrations of NH_4^+ can be produced by bacterial ureolysis (according to Eq. 5.1). For precipitation to occur, the ion activity product (IAP) must be greater than the ion activity product at equilibrium in a solution with struvite (K_{sp}). The activity of a species is an effective concentration, which takes into account interactions with other species. Thus, in urine where other ions are present, they will interact with each other and change the "effective concentration" of neighboring ions. Because activity is based on a ratio comparing concentration to the pure species concentration, it is dimensionless by convention. In very dilute solutions, activity is well-approximated by concentration. If the IAP is greater than the K_{sp}, the **saturation index** (Eq. 5.3) is positive and the solution is called supersaturated. For struvite at body temperature, the K_{sp} value is approximately 10^{-13} [13]. Supersaturation implies that precipitation is possible, but we must consider that slow rates of precipitation or molecules that inhibit precipitation (e.g., proteins or polysaccharides that either block surfaces or sequester ions from solution) can allow a solution to remain supersaturated on practical time scales without precipitation. In other words, supersaturation is necessary for precipitation, but does not guarantee that it will occur.

$$Mg^{2+} + NH_4^{+} + PO_4^{3-} + 6H_2O \leftrightarrow MgNH_4PO_4 \cdot 6H_2O\left(\text{struvite}\right) \tag{5.2}$$

$$\text{Saturation Index} = \log\left(\text{IAP} / K_{sp}\right) \tag{5.3}$$

Example Problem

At a location in the urinary tract, the struvite ion activity product (IAP) is 10^{-12}. If the K_{sp} of struvite at body temperature (37 °C) is 10^{-13}, is the solution

supersaturated? If struvite does not precipitate in this system what are some possible explanations?

ANSWERS: Yes, the solution is supersaturated. Precipitation could not occur because

(a) There are inhibitors that sequester ions or lower their activity.
(b) The kinetics are too slow relative to the residence time of the system.

The mechanism of struvite stone formation starts with nucleation and proceeds via layer-by-layer crystal growth along with the aggregation of crystals. The precise mechanisms of nucleation and growth are controlled by the saturation index, impurities, and surface structures. For a detailed description of crystal growth and nucleation, the interested reader is referred to De Yoreo and Vekilov [14]. Conceptually, it is important to keep in mind that, very likely, all nucleation in the urinary tract occurs on pre-existing surfaces (i.e., heterogeneous nucleation) rather than in solution (i.e., homogeneous nucleation). In other words, nearly all crystals will be associated with surfaces, but these surfaces could be from suspended particles in the bulk fluid (e.g., bacteria, proteins, dead human cells, etc.), and not only the surface of the ureter, catheter, etc.

Biofilms can promote the aggregation of small crystals to form larger stones. For this reason, infection stones are known to grow exceptionally fast compared to metabolic stones [15]. Furthermore, the shape and properties of struvite stones are influenced by the organic molecules that are incorporated within the stones, such as polysaccharides, mucoproteins, and glycosaminoglycans [16]. Treatment methods aimed at breaking up or dissolving infection stones must therefore consider the biomolecules integrated within the stones.

Techniques to Investigate Struvite Stone Formation In Vitro

Mineral precipitation and biofilm formation are governed by micro- and nano-scale processes that are difficult to observe *in vivo*. Until recently, our knowledge of stone formation and treatment has relied primarily on clinical observations such as treatment efficacy and patient history. But in order to understand the underlying processes, clinical-based hypotheses must be tested in controlled experiments. Laboratory (*in vitro*) systems and computer (*in silico*) simulations offer advantages for studying stone formation. Noninvasive clinical observations are generally limited to urine analysis and ultrasound, MRI or CT imaging which do not have the spatial and temporal resolution required to study microbe-induced stone formation in detail. Furthermore, ethical issues prevent many types of controlled *in vivo* experiments (e.g., varying pH, the addition of non-clinically-approved compounds or the use of destructive imaging techniques).

Forming stones in artificial environments, or *in vitro*, provides an opportunity to study individual parameters that might affect stone formation. The simplest *in vitro* experiments are conducted in batch cultures under static [17] or stirred [18] conditions

with synthetic or real urine. Batch studies have been useful in screening for potential urease inhibitors [18] or testing different stent materials for their resistance to encrustation [19, 20]. However, batch experiments cannot simulate the flow conditions that exist in much of the upper renal tract. Continuous flow systems are useful for testing ureteral stents and for the development and testing of antimicrobials or precipitation-inhibiting materials [21]. One standardized method to test urological device materials uses a CDC Biofilm Reactor (ASTM E2562, 2007) [22]. The CDC Biofilm Reactor allows for biofilms and minerals to be formed on removable coupons in a well-controlled biochemical environment, thus allowing for the study of the influence of different microbes, metabolisms and urine chemistries. Other specialized reactors have been developed to more closely model the bladder [23].

Modern software for **reactive transport modeling** has made it easier to conduct experiments *in silico*. These simulations supplement laboratory studies and can guide future experiments and treatment strategy development. When modeling precipitation in urinary tract systems, there are three interconnected factors that must be considered: (1) diffusion, (2) reaction and (3) fluid flow. Additionally, solid mechanics (tissues) and viscoelasticity (biofilm) can be considered in the models.

Figure 5.2 illustrates how fluid flow and chemistry vary spatially. A fluid velocity field can be calculated based on the geometry of the system. Ureolysis depletes urea locally, producing ammonium and affecting the local saturation index of struvite as illustrated in Fig. 5.2. Ammonium concentrates within the biofilm and diffuses into the bulk fluid due to the concentration gradient, which can affect the saturation state downstream of the ureolytic biofilm. Although Fig. 5.2 shows a qualitative demonstration, commercial finite element software is available for accurate quantitative analyses. Finite element models split larger problems (i.e., large geometrical domains) into a system of smaller problems (elements) that can be solved and reconstructed. COMSOL Multiphysics (COMSOL Inc., USA) is one example of a user-friendly and flexible finite element modeling program that is capable of modeling all of the processes mentioned in this chapter. Studies by Bucs et al. [24] and Radu et al. [25] provide good examples of COMSOL being used to model biofilm growth and mineral precipitation.

Predicting an accurate saturation index is not straightforward in a complex liquid like urine, but **geochemical equilibrium modeling** software can make this easier. The influence of solid phases can be predicted, as well as the speciation of magnesium, phosphate and nitrogen, which are affected by fluid conditions and equilibria with other ions. For example, the dissolved species affecting struvite precipitation include at least $H_2PO_4^-$, H_3PO_4, OH^-, NH_3, $MgOH^+$, $MgPO_4^-$, $MgH_2PO_4^+$, $MgHPO_4$, HPO_4^{2-}, Mg^{2+}, PO_4^{3-}, and NH_4^+.

A popular open source geochemical modeling software is PHREEQC [26], however other programs exist. Even though these geochemical models were developed for nonmedical purposes, such as groundwater chemistry modeling, they are useful for modeling stone formation. Effective geochemical modeling can also be coupled with or incorporated into finite element models for a more thorough description that accounts for location and fluid flow in organs of the urinary tract [27].

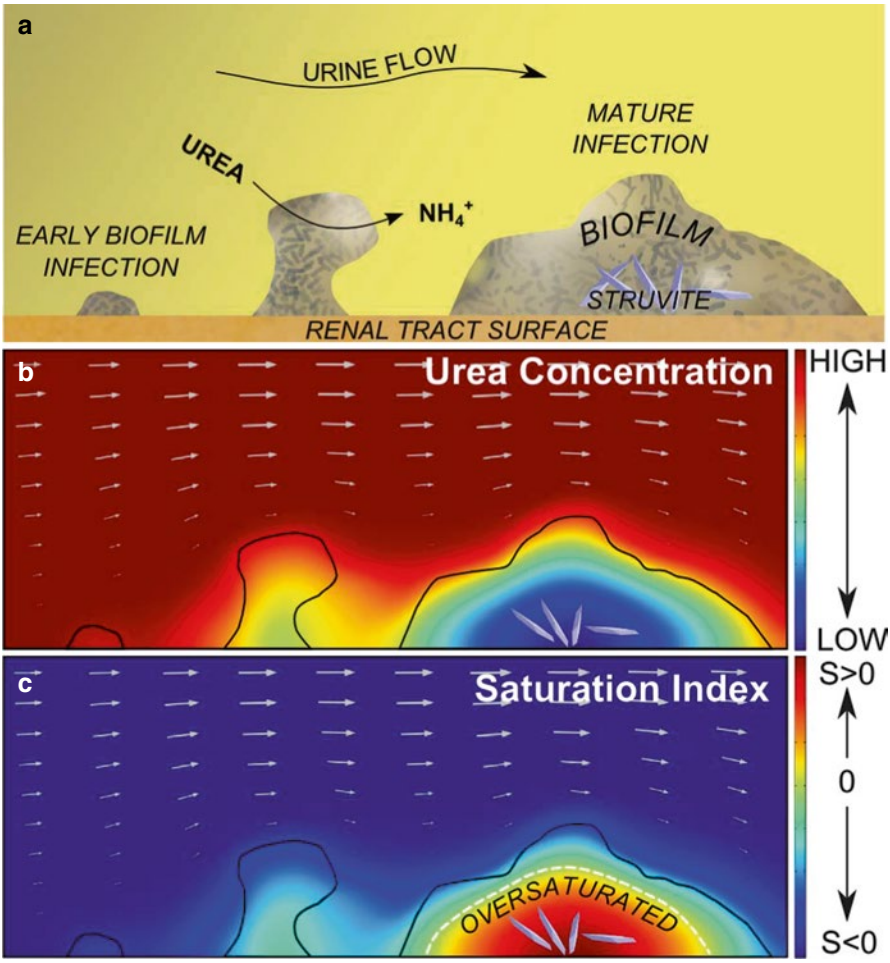

Fig. 5.2 (**a**) Cross section of a ureolytic biofilm on a renal surface. (**b**) Ureolysis occurs within the biofilm decreasing the concentration of urea inside biofilms as the biofilm gets thicker and more mature. (**c**) The saturation index increases within the biofilm due to ammonium production. Thicker biofilm will result in a more saturated environment where struvite precipitation is more likely to occur

Clinical Treatment of Struvite Stones

When struvite stones form and disrupt the urinary tract, there are three treatment steps: First, the urinary tract must be disinfected, typically through antibiotic treatment. Second, the stones must be carefully removed, typically by surgery. Finally, recurrence must be prevented. Generally it has been shown that lowering the pH of urine (acidification) and reducing the concentration of precursors such as calcium and magnesium, reduces stone formation [28, 29]. Urease inhibition has been

proposed since the early 1960s, specifically the use of hydroxamic acids such as aceto-hydroxamic acid, which causes irreversible, non-competitive inhibition of urease – other urease inhibitors include hydroxyurea, biosuppressin, and furosemide [5]. But in many cases, neurological, dermatological, or hematological side effects occur, so clinical approval has been hampered. A more complete description of struvite stone treatment is discussed in Chap. 6.

Summary

In the renal tract of a healthy individual, struvite ($MgNH_4PO_4 \cdot 6H_2O$) stones rarely form spontaneously. When a urinary tract infection occurs, bacteria that use urease to break down urea ($CO(NH_2)_2$) into ammonium (NH_4^+) affect the chemistry of urine and can induce struvite precipitation. Infections are often – but not always – correlated with implanted devices, and the resistance of the microbial infections to traditional treatments (such as antibiotics) is aided by the existence of attached communities known as biofilms. Modern laboratory systems and computer models are improving our understanding of how and why infection stones such as struvite form. A better understanding of the complex microbe-fluid-mineral interactions will lead to less invasive treatments and more effective prevention strategies.

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Chapter 6
The Management of Infection Stones

Manoj Monga and Sarah Tarplin

Abstract "Infection stones," or stones formed as a result of urinary tract infection (UTI) with a urease-producing organism, are more common in women and those with functional or anatomic abnormalities of the urinary tract. Infection stones may form staghorns and the prognosis is poor if left untreated. The primary therapeutic goal should be that the patient is stone-free. To that end, percutaneous nephrolithotomy (PCNL) or other modern endoscopic interventions, in addition to culture-specific antimicrobial therapy are required. Hydroxamic acid and long-term antibiotic therapy may be useful adjuncts in some patients. Sepsis following PCNL is an ongoing challenge and can occur despite prophylactic antibiotic administration and treatment of a positive pre-operative urine culture. Recent studies suggest that a longer course of antibiotics prior to PCNL may be beneficial in some patients. Stones formed due to a metabolic derangement can lead to infection by providing a nidus for bacterial growth or by causing obstruction. After collecting system decompression and delayed stone removal, it is important to identify and manage all risk factors for infection in patients with stones and concomitant infection.

Introduction to Infection Stones

Nephrolithiasis and urinary tract infection should be compartmentalized into two different entities with different pathophysiology and management strategies. However, at times their pathophysiology and management plans overlap. "Infection stones" are caused by recurrent infection with a urea-splitting organism. Conversely, "stones with infection" are stones formed as a result of a metabolic abnormality that can lead to infection due to urinary obstruction [4, 5].

Struvite stones, or "infection stones," comprise 5–15 % of all renal calculi [20]. Struvite stones are associated with both significant short-term and long-term

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morbidity; complications include recurrent urinary tract infections (UTIs), pain, pyelonephritis, hydronephrosis, and perinephric abscess formation [20, 23]. The prognosis of the untreated staghorn calculus is dismal; it may result in renal deterioration and in rare cases, death [23]. In one study, at 8 years follow-up, 67 % of those who refused surgery suffered a renal-related death [44]. Despite treatment with percutaneous nephrolithotomy (PCNL), a significant proportion of patients with struvite stones develop worsening renal function and renal failure [21, 44]. The recurrence rate and high risk of long term, serious sequela mandates prompt intervention, careful microbial investigation, and tailored preventive care for patients upon their first stone episode.

Pathophysiology of Struvite Stones

Struvite stones are comprised of magnesium ammonium phosphate. The stone propagates with time, forming staghorn calculi, branched stones that fill a portion or the entirety of the renal collecting system. While staghorn stones usually consist of a combination of struvite and calcium carbonate apatite, other components, such as uric acid and cystine are not uncommon [34, 39]. The development of the struvite stone begins with infection of the upper urinary tract with a urease-producing organism, such as *Proteus, Mycoplasma, Providencia*, or *Staph Aureus*. The urease breaks down urea into ammonia and carbon dioxide; ammonia then reacts with water, producing ammonium and subsequently alkalinizing the urine. This high pH decreases the solubility of phosphate in urine and promotes the crystallization of magnesium ammonium phosphate [17, 34].

The formation of struvite stones is gender-specific; women are 1.6× more likely than men to develop struvite stones [8]. Those with functional or anatomic abnormalities of the urinary tract are more prone to struvite calculi. For example, patients with urinary diversion and urinary reservoirs containing intestinal segments are more likely to develop calculi due to increased mucus production, bacterial colonization, and chronic urinary stasis. In one study, struvite calculi were the most common type of calculi found in patients with intestinal and gastric reservoirs at an average of 3.6 years post augmentation cystoplasty [22]. 3.5 % of patients with spinal cord injury (SCI) develop struvite stones, likely due to neurogenic bladder dysfunction involving poor bladder compliance, high detrusor pressures and insufficient bladder emptying. Additionally, Vesico-ureteral reflux (VUR) and indwelling catheters have been associated with increasing the risk of struvite stones in the setting of SCI [10]. Although current urologic techniques and urodynamics have improved the management of voiding dysfunction and chronic UTI, struvite stones present an ongoing challenge for patients with SCI [10, 19]. In SCI patients, struvite stones may present with recurrent UTIs or urosepsis, and struvite stones form staghorns in 30 % of cases. Interestingly, a large portion of these patients do not develop their first stone until many years after spinal cord injury [10].

Stones with Infection

In the setting of infection stones, the urease-splitting infection itself is the primary derangement promoting the development of the struvite stone, which must be distinguished from stones complicated by infection. In the case of a "stone with infection," a stone of metabolic origin can lead to UTI by causing obstruction or by providing a nidus for bacterial growth. In this case, a patient may present with pyelonephritis or urosepsis necessitating urgent intervention, and others may be asymptomatic, with their only finding being asymptomatic bacteriuria. In the situation of infection with stones, the impact of stone extraction on the subsequent risk of future urinary tract infections is poorly defined.

Perioperative Infection in the Setting of Stone Surgery

Managing the risk of serious infection following stone surgery is a challenging task. Sepsis remains a life-threatening problem following stone surgery even in patients with sterile urine and in the presence of prophylactic antibiotics. In fact, 37 % of patients develop SIRS (systemic inflammatory response syndrome) criteria following PCNL [25]. This can be attributed to a variety of factors, including upper tract manipulation, urinary obstruction, and bacteria or endotoxins (produced by bacteria) present within the stone [42]. Importantly, routine mid-stream bladder culture has not been predictive of this risk of SIRS, and anti-microbial treatment of a positive pre-operative urine culture does not necessarily lessen the risk of sepsis [13, 25]. This may be a result of pre-operative treatment targeted at the wrong pathogen. Furthermore, it is important to obtain urine samples for culture from the appropriate sites. In a study by Mariappan et al., patients with positive stone culture or positive renal pelvis culture were four times more likely to develop SIRS following PCNL. Additionally, it was demonstrated that while positive urine cultures from the renal pelvis effectively predict stone infection (defined by positive stone cultures), positive bladder cultures do not [25]. In fact, culture and sensitivities obtained from the bladder may reveal different microbial flora than the upper tract. This may explain why patients re-admitted for sepsis following stone surgery often grow out a different pathogen that the pathogen seen on the preoperative urine culture [11]. In light of these findings, some may recommend obtaining routine pelvic urine and stone cultures at the time of PCNL [25].

Prophylactic Antimicrobial Approaches

Currently, there is no universal consensus regarding the optimal antimicrobial approach to patients undergoing percutaneous renal surgery. Most institutions routinely implement the use of prophylactic antibiotics at the time of surgery in the

presence of sterile urine. Although the American Urological Association (AUA) asserts that there are no randomized controlled trials clearly outlining the need for antimicrobial prophylaxis during percutaneous renal procedures, the best practice policy statement recommends prophylaxis with either a first or second generation cephalosporin or a combination of an aminoglycoside plus metronidazole or clindamycin [48]. Alternatives include ampicillin/sulbactam or a fluoroquinolone. A single dose has been shown to have the same effect as the continuation of therapy until nephrostomy tube removal [9, 48].

There is also some controversy regarding appropriate treatment of a positive urine culture in the setting of PCNL. The Infectious Diseases Society of America (IDSA) recommends a specific approach to the management of patients with asymptomatic bacteriuria in the setting of urologic surgery. The IDSA recommends antibiotic prophylaxis for persons undergoing any urologic procedures that have the high potential to cause mucosal bleeding. Although these recommendations are based on randomized trials of men undergoing transurethral resection of the prostate, there is a concern for high rates of sepsis in any urologic procedure causing mucosal trauma [31]. For these patients, the IDSA recommends the evidence-based initiation of antibiotic prophylaxis the night before surgery or at the time of the surgery [1, 6, 31]. Here, other parameters, such as the most appropriate time to draw the cultures and the exact duration of the antibiotic therapy, are not explored and remain poorly defined. The IDSA suggests that for most patients, it is appropriate to discontinue antibiotics after the procedure, although there is no evidence for this specifically in cases of stone surgery [1, 6, 31].

Prolonged Antibiotic Regimens

Unfortunately, urosepsis after PCNL is an alarming reality even in the presence of sterile mid-stream urine cultures and this type of routine antibiotic prophylaxis. Rates of sepsis following PCNL have doubled from 1999 to 2009 and a startling 4 in 1000 patients undergoing PCNL die [14]. Several relevant risk factors for urosepsis in this setting have been described in the literature, suggesting that this limited antibiotic regimen may not be appropriate for everyone. Those with indwelling nephrostomy tubes, bladder outlet obstruction, and positive preoperative urine cultures are at a higher risk of sepsis, although treatment of the positive culture does not necessarily reduce the rate of sepsis [13]. Sepsis is associated with prolonged operative times, degree of obstruction in the urinary tract and the presence of bacteria in the upper tract. Here, the mechanism is related to leakage of endotoxins and bacteria into the blood [2, 18, 32, 35]. Also, larger stones have been shown to harbor higher levels of endotoxins, and have been linked to higher risks of infection and postoperative urosepsis [25–27, 29].

One non-randomized, controlled trial addressed the need for a prolonged duration of antibiotic prophylaxis in patients harboring these higher risk stones. Mariappan et al. [26] examined the use of a regimen of ciprofloxacin 250 mg twice a day for 7 days prior to PCNL for patients with dilated pelvicalyceal systems and

stones ≥2 cm. The study found that this regimen significantly reduced the rates of upper tract UTI, SIRS, and urosepsis compared with controls, suggesting that those at higher risk for urosepsis due to obstruction and large stone burden may benefit from an extended course of antibiotics prior to stone surgery [26]. Similarly, in a prospective randomized, controlled study, Bag et al prescribed patients with hydronephrosis and/or stone >2.5 cm a 1 week long regimen of nitrofurantoin prior to PCNL, and found significantly lower rates of endotoxemia and urosepsis [2]. In light of these findings, one option is to tailor the antibiotic regimen to each patient by prescribing 1 week of antibiotics only to patients with certain risk factors, such as indwelling catheters, neurogenic bladder, recurrent infections, obesity, stones >2 cm, hydronephrosis,, and struvite stones [2, 26]. Antibiotics may also be administered to patients with pyuria even if the culture is negative. In patients with these risk factors for sepsis, antibiotic regimens could be further tailored according to patients' previous microflora and antibiotic use; local antibiograms may also guide the choice of antibiotic. Alternatively, it is reasonable to prescribe 1 week of antibiotics (i.e., ciprofloxacin or nitrofurantoin) to all patients undergoing percutaneous nephrolithotomy.

Treatment of Struvite Stones: Strategies and Medical Management

In the treatment of patients with struvite stones, the primary, evidence-based therapeutic goal should be that the patient is stone-free. It has been demonstrated that complete elimination of the stones combined with appropriate antimicrobial therapy can decrease the stone recurrence rate to only 10 %; conversely, retention of stone fragments can result in a recurrence rate of up to 85 % [46]. Contemporary surgical interventions, such as PCNL or other endoscopic interventions should be performed as first-line treatment toward this end goal. Conservative, non-operative therapy is not recommended as the sole treatment for staghorn calculi in most patients, as it is highly unsuccessful, and has a well-established risk of renal failure in the long-term [23, 44]. However, medical therapy with urease-inhibitors such as hydroxamic acid is a useful option for the treatment of residual struvite stone material when it cannot be completely eradicated. The most relevant substance, aceto-hydroxamic acid (AHA) serves as a non-competitive inhibitor of urease, lowering the pH and ammonia levels and augmenting the efficacy of some antibiotics [3, 49]. In randomized, placebo-controlled trials, AHA was shown to hinder or prevent further struvite stone growth in patients infected with urease-producing bacteria [15, 16, 47, 49]. However, AHA has several limitations. It is contraindicated in patients with serum Cr > 2.5 mg/dl and it is a known teratogen. Bothersome adverse effects, such as nausea, vomiting, headache, tremulousness, and anxiety are very common and often intolerable for patients, often requiring discontinuation of the drug [47, 49]. Additionally, there is a 15 % risk of deep vein thrombosis [12]. Patient populations with the highest stone recurrence rate, such as those with neurogenic bladder or urinary diversion may be

appropriate candidates for AHA [43]. In addition, AHA is a viable option in those who cannot tolerate or refuse operative intervention, or if anatomical variations prevent the stone from being accessed by traditional endoscopic techniques. When indicated, AHA is initiated at 250 mg twice a day, and may be titrated up to 250 mg 3–4 times a day [49]. The long term efficacy in deterring stone growth and long-term safety of AHA are not fully outlined.

Chemolysis may be an option in select cases of patients who are not surgical candidates. This involves irrigation of the collecting system through a nephrostomy tube or ureteral catheter with Renacidin [3, 34]. Renacidin has been used historically to dissolve stones with success rate of 65–85 % and may aid in the dissolution of stones following PCNL [3, 30]. Renacidin requires further hospitalization, and is associated with significant adverse effects [34]. Currently, Renacidin irrigation preparations are no longer manufactured in the U.S.

The Role of Antimicrobial Therapy in the Treatment of Struvite Stones

Culture-specific antibiotics are important in the preoperative and perioperative period to prevent sepsis. Antimicrobial therapy targeting the urease-producing bacteria decreases the production of ammonia, decreasing the supersaturation of struvite and apatite in the urine, therefore lowering the risk of stone formation and preventing further stone propagation [3]. Antibiotics alone are not sufficient to treat infection and do not usually sterilize urine in the setting of the struvite stones, as the medication cannot reach the bacteria, which reside deep within the stone. The use of suppressive antibiotics for patients with struvite stones has been proposed as a method of suppressing remaining bacterial activity [30]. Long-term, culture specific antibiotics are capable of diminishing the urinary tract infection although they may not necessarily produce sterile urine. Overall urease activity can be decreased by 99 % by lowering colony counts from 10^7 to 10^5 with antibiotics [46]. Long-term, low dose antibiotics selected according to the bacterial culture and sensitivities may hinder stone growth in the case of recalcitrant stone fragments after surgery or when surgery is not feasible. Yet the role of suppressive antibiotics is limited by the possibility of developing multi-drug resistant bacteria [33]. Importantly, targeted, long-term antibiotics do not work to dissolve the stone, but rather to impede further growth and recurrence of an existing stone [46]. Currently, there are no randomized controlled trials in humans clearly demonstrating the beneficial role of suppressive antibiotic therapy and it is not universally recommended to all struvite stone formers [3]. According to AUA recommendations, patients with predominantly struvite/carbonate apatite stones may be candidates for such suppressive therapy due to the persistent, increased risk for recurrent UTIs despite stone removal [34]. In such cases, one option is to initiate long term antibiotics at therapeutic doses, followed by a switch to suppressive doses once urine sterility is achieved. It is helpful to follow-up with monthly urine cultures to track asymptomatic bacteriuria.

Treatment of Stones with Infection

As previously mentioned, it is common for a stone to facilitate urinary tract infection by providing a nidus for bacterial proliferation. In patients with an obstructing calculus and concomitant infection, a ureteral stent or nephrostomy tube should be placed promptly for collecting system decompression and stone removal surgery should be delayed. Modern endoscopic interventions such as ureteroscopy or PCNL are routinely used several weeks after the fulminant infection has been eradicated.

In patients with stones and concomitant UTIs, it is important to identify and treat other potential risk factors for infection. Women are most commonly affected by recurrent infections due to ascent of bowel organisms [40]. Any patient with upper tract infection, renal calculi, signs or symptoms of fistula, urinary obstruction, bladder dysfunction, hematuria after resolution of infection, or history of abdominal or pelvic malignancy should undergo individualized urologic evaluation [7, 40]. Any abnormality that alters the urogenital flora, or reduces the production, flow, or complete emptying of urine can predispose the patient to reinfection [40]. Postmenopausal women are at increased risk for reinfection, as the paucity of estrogen changes the urogenital flora, rendering the patient susceptible to E. Coli [40]. Estrogen replacement has been used successfully as a method of restoring the commensal lactobacilli and has been shown to decrease the rates of urinary tract infection [36, 40]. Both topical estrogen creams and rings are acceptable options, depending on patient preference. In addition, the post-menopausal patient may have some degree of impaired emptying due to cystocele or pelvic prolapse, leading to residual urine which serves as a nidus for bacterial colonization [40]. Surgical and medical management of pelvic floor abnormalities and other disorders with residual urine is essential in the treatment of those with recurrent infections.

Prevention of Infection Stones

The prevention of the struvite stone is an integral part of care, since the risk of stone recurrence is high, especially in the setting of staghorn calculi [43]. In the case of staghorn calculi, stone analysis is an important initial measure. Although staghorns are usually comprised of struvite/calcium carbonate apatite, other stone components may be present, and abnormal levels of urinary constituents may contribute in part to the overall pathophysiology. There is ongoing controversy regarding prevalence of abnormal metabolic findings in patients with struvite stones. The older literature supports the importance of metabolic investigations in patients with infection stones, demonstrating that metabolic abnormalities are relatively common [37, 38]. In many of these studies, the authors do not distinguish between pure struvite versus mixed stones, and there is a lack of clarity in physician definition of “infection stones.” Others propose that patients with pure struvite/calcium carbonate apatite stones do not require 24 h urine or metabolic evaluation, demonstrating that metabolic derangements relating to stone formation are rarer in these patients [24, 34].

As a part of a thorough work-up, 24 h urine collection studies should be considered in patients with both pure struvite stones and mixed stones. These studies may reveal underlying, treatable metabolic abnormalities contributing to renal calculi formation [49].

Streem et al demonstrated that the 5 year risk of struvite stone recurrence after treatment is 37.8 %. Thus, most patients require consistent, long-term surveillance and appropriate methods to eliminate all stone material, prevent further stone formation, and quell persistent infection [46]. Urine screening is important to identify cases of persistent infection. After culture-specific antibiotic therapy, monthly follow –up visits with urine culture for the first 3 months are important, followed by repeat urine cultures every 3 months for all struvite stone formers [3, 46]. Annual surveillance renal ultrasound is recommended, especially in those at high risk for recurrence, such as patients with spinal cord injury [33]. Patients with spinal cord injury require an appropriately long duration of follow-up, as patients often present with their first stone episode many years after their injury [10]. Additionally, AHA can be utilized in patients with residual stone fragments as tolerated. The approach of suppressive antibiotics for patients presenting with recurrent UTIs in the setting of struvite stones is controversial and requires further investigation, but should be considered in select patients [33]. Other approaches include adequate fluid intake (2.5–3 L/day) and minimizing the use of indwelling catheters. In fact, improvements in bladder management strategies and less reliance on indwelling catheters for patients with neurogenic bladder have led to a shift in the composition of stones in this population from struvite to predominantly metabolic [28].

Prevention of Stones with Infection

Behavioral interventions such as adequate fluid intake to promote diuresis and regular voiding can be useful adjuncts in preventing urinary tract infections. Frequent voiding can diminish the incubation period of the bacteria in the bladder, thereby impeding the establishment of infection [45]. Adequate anti-microbial prophylaxis is an integral part of preventing catastrophic infection in recurrent stone formers. Women with greater than two symptomatic urinary tract infections within 7 months or greater than three in 12 months can be considered for prophylactic antibiotics [40]. One strategy is a continuous, low dose antibiotic. The goal is to eradicate uropathogens from the bowel and urogenital area while limiting the evolution of resistant organisms. Certain regimens have well-documented efficacy in prophylaxis. TMP-SMX (Bactrim) works to clear pathogenic gram negative aerobes from the rectal and vaginal environment. Nitrofurantoin reaches intermittent, brief peaks in the urine, and does not affect the bowel microenvironment, therefore allowing colonization by bowel pathogens but preventing them from establishing infection [40, 41]. Finally, cephalexin at low doses (250 mg or less) has been shown to produce sterile urine without altering the bowel flora or leading to resistant organisms [40]. Thus, prophylaxis should be initiated with either nitrofurantoin, at 50–100 mg half

strength, TMP-SMX, at 40–200 mg, or cephalexin, at 250 mg. These therapies have been shown to decrease recurrences of UTIs by 95 % [40, 41].

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Chapter 7
The Use of Probiotic Bacteria to Treat Recurrent Calcium Oxalate Kidney Stone Disease

Brian R. Kullin, Sharon J. Reid, and Valerie R. Abratt

Abstract Calcium oxalate-based kidney stones are the most common type found amongst idiopathic stone-forming patients. Excess dietary oxalate can be excreted via the faeces as well as the urine, and consumption of oxalate degrading probiotic bacteria might assist in reducing hyperoxaluria by degrading dietary oxalate in the gastrointestinal tract (GIT) before it can be absorbed. This chapter describes the genetic and *in vitro* aspects of microbial oxalate metabolism, and reviews *in vivo* trials involving the use of specific probiotic bacteria. Recent novel approaches using ingested purified oxalate degrading enzymes or *in vivo* expression of recombinant enzymes to reduce hyperoxalauria are also discussed.

In vitro studies have shown that certain *Lactobacillus* and *Bifidobacterium* species may have great potential for use as oxalate degrading probiotics since they reduce oxalate but can also survive in the gut under conditions where oxalate is limited. Gut colonisation and *in vivo* bacterial oxalate utilization studies in humans have shown a similar trend towards reducing oxalate levels. However, most of these interventions have been limited in their scope and need more rigorous investigation to measure their therapeutic value. A recent alternative approach used known amounts of *in vitro* purified recombinant oxalate decarboxylase enzyme to treat hyperoxaluria in animal models. These showed urinary oxalate degradation and low toxicity. Rats colonised with *Lactobacillus plantarum* expressing this recombinant enzyme also showed a significant reduction in urinary oxalate. The approaches reviewed here show potential therapeutic value *in vitro*, but all require extensive further evaluation in well-designed human trials.

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Introduction

Kidney stone disease affects between 5 and 20 % of people worldwide [43]. Adding to the disease burden is the high rate of stone recurrence, with up to half of stone formers going on to experience further stones within 10 years of their first episode [30]. Recent estimates put the cost of stone disease in 2007 at $3.79 billion in the US alone, a figure which is expected to continue to rise to >$5 billion per year by 2030. Prophylactic treatment strategies that result in a reduced risk of stone formation and recurrence are an economically attractive option [7].

The Importance of Calcium and Oxalate in Kidney Stone Disease

Calcium oxalate-based kidney stones are by far the most common form amongst idiopathic stone-forming patients [57], and increased urinary concentrations of both calcium and oxalate are major risk factors for stone formation. Urinary calcium is predominantly dietary in origin, although bone calcium may be an important contributor in individuals on low-calcium diets [12]. In contrast, urinary oxalate is mainly the result of endogenous metabolic processes, with an additional contribution from the consumption of oxalate-containing foods [27]. Efforts to reduce kidney stone recurrence through dietary modulations have generally focussed on lowering the amount of oxalate consumed rather than limiting calcium intake. However, maintenance of sufficient dietary calcium is important in lowering oxalate assimilation in the gastrointestinal tract (GIT) because it binds to oxalate to form insoluble calcium oxalate, which is subsequently removed via the faeces [40, 53].

An adjunct approach to lowering the level of urinary oxalate is to consume probiotic bacteria, which have the ability to degrade dietary oxalate in the GIT before it can be absorbed. This chapter will focus briefly on the genetic and *in vitro* aspects of microbial oxalate metabolism, followed by the evidence from *in vivo* trials involving the use of probiotic bacteria, and finally some of the recent novel approaches, using recombinant enzymes.

Microbial Colonisation of the Gut

The human GIT is colonised by large numbers of different bacterial species [32], with the major profiles of the microbiota being particular to an individual. These microbial profiles are generally stably maintained, but can vary to an extent over time within individuals in response to diet and other factors such as ingesting antibiotics and potentially toxic compounds such as oxalate [21]. GIT bacteria carry out a range of biochemical reactions which can affect human health and nutrition and can degrade many dietary substances that cannot be digested by humans [20]. Some

species have been used extensively as probiotics in foodstuffs and pharmaceuticals since colonisation of the gut by these beneficial bacteria may contribute to human health in various ways. Recent studies show that certain bacterial genera can utilise the oxalate present in the gut lumen, potentially preventing absorption into the bloodstream and subsequent excretion in the urine [33].

Genetic Basis of Bacterial Oxalate Metabolism

Of the various GIT bacteria involved in oxalate catabolism, the most widely studied are *Oxalobacter formigenes* and a range of other bacteria belonging to the group generally called the lactic acid bacteria (LAB) particularly, members of the *Lactobacillus* and *Bifidobacterium* genera. These will be reviewed here.

Oxalobacter formigenes

Much of the early research regarding the genetics of microbial oxalate metabolism was carried out on the 'specialist oxalotroph', *O. formigenes* (discussed in more detail elsewhere in this publication), which requires the presence of oxalate for growth. When used in the production of energy, intracellular oxalate is metabolised by a two-step process whereby it is first activated through the addition of a coenzyme A moiety and then decarboxylated to produce formyl-CoA and CO_2 (Fig. 7.1). The enzymes responsible for these two reactions are encoded by the *frc* (formyl-CoA transferase) and *oxc* (oxalyl-CoA decarboxylase) genes [9, 10, 31, 48]. The exchange of extracellular oxalate^{2-} and intracellular formate^{1-} (via the OxlT transporter), along with the consumption of H^+ ions during the decarboxylase reaction, allow the production of ATP by a membrane bound F_0-F_1-ATPase [1, 5, 14, 25, 44].

Lactobacillus spp. and *Bifidobacterium* spp.

Perhaps the candidates with the greatest potential for the treatment of kidney stone disease are the oxalate-degrading *Lactobacillus* and *Bifidobacterium* spp. [2]. These genera have a long history of use as probiotics and many species have been granted 'Generally Regarded as Safe' (or 'GRAS') status, which facilitates their use commercially. They appear to degrade oxalate through a similar mechanism to *O. formigenes*, possessing both *oxc* and *frc* genes. However, the mechanism of oxalate uptake for these genera has not yet been elucidated. It is interesting to note that *Lactobacillus* and *Bifidobacterium* spp can grow on a range of alternate energy sources in addition to oxalate and are, therefore, called "generalist oxalotrophs". This allows them to survive in the gut even when oxalate levels are low.

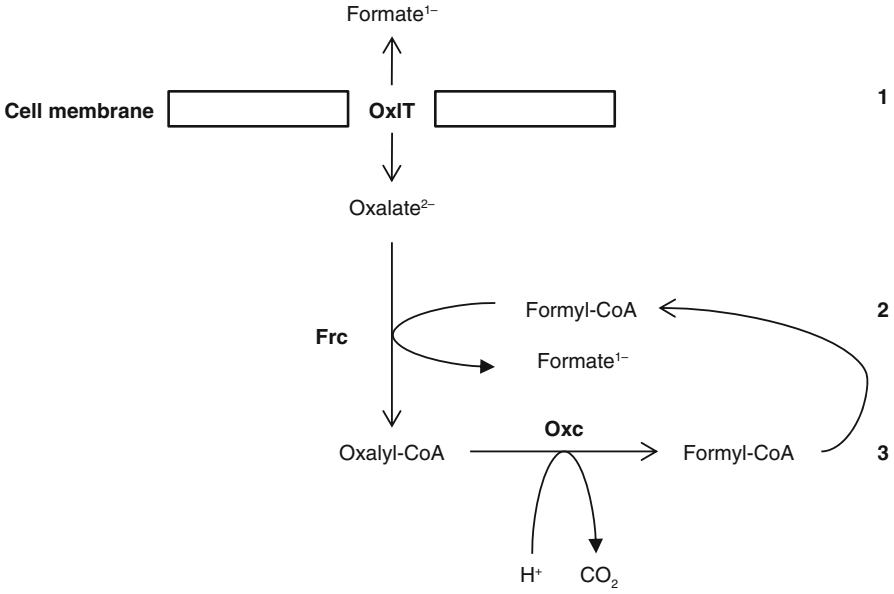

Fig. 7.1 Schematic diagram of oxalate utilisation by *O. formigenes*. (*1*) Oxalate^{2-} enters the cell via an oxalate:formate antiporter (*OxlT*). (*2*) Oxalate^{2-} is activated by the transfer of CoA from a formyl-CoA donor in a reaction catalysed by a formyl-CoA transferase (*Frc*). (*3*) Oxalyl-CoA is decarboxylated by an oxalyl-CoA decarboxylase (*Oxc*)

The majority of genetic research thus far has focussed on *Lactobacillus acidophilus* and *Lactobacillus gasseri*. In both species the *frc* and *oxc* gene homologues are situated adjacent to one another on the genome, unlike the arrangement in *O. formigenes* where the genes are located separately [8, 26, 55]. Importantly, expression of both genes requires mildly acidic (pH 5.5) growth conditions, similar to those present in the lower GIT. It is possible that one or both of the gene products function during the bacterial response to acid stress as the consumption of H^+ ions during oxalate degradation can help to buffer the internal pH of the cell. This is seen in experiments using *L. acidophilus*, where inactivation of the *frc* gene resulted in increased susceptibility toward oxalic acid [8].

Bifidobacterium animalis subsp. *lactis* is the only member of the bifidobacteria for which a functional characterisation of the genes involved in oxalate degradation has been carried out. Screening of a *B. animalis* subsp. *lactis* genomic library using a probe prepared from the *oxc* gene from *O. formigenes* allowed the identification of a *oxc* homologue [15]. Later studies identified a putative *frc* homologue as well as a conserved hypothetical protein, which may function as an oxalate transporter [54]. As for the lactobacilli, acidic conditions were required for expression of the *frc* and *oxc* genes, although in this case a lower pH was required (pH 4.5). Recently published genome sequences have revealed that putative *frc* and *oxc* genes are

present in several additional *Bifidobacterium* spp., including *B. dentium*, *B. gallicum*, *B. pseudocatenulatum* and *B. pseudolongum*, however, functional characterisation of the genes is lacking in these species.

In Vitro and Animal Models for Oxalate Utilization by GIT Bacteria

The *in vitro* oxalate degradation capacity of the various *Lactobacillus* and *Bifidobacterium* spp has been shown to be both species and strain specific. Mogna et al. [36], demonstrated that the best oxalate-degraders (68 %) in the *Lactobacillus* group came from *L. paracasei*, *L. gasseri* or *L. acidophilus* while Turroni et al. [55] isolated a range of novel *Lactobacillus* spp (including *L. acidophilus* and *L. gasseri*) that could degrade more than 50 % of the oxalate present. In the *Bifidobacterium* group, Federici et al. [15] reported that the highest level of oxalate degradation was with *Bifidobacterium lactis* DSM 10140 at 61 %, while *Bifidobacterium longum* MB 282 and *Bifidobacterium adolescentis* MB 238 showed 35 % and 57 % degradation respectively. In general, all the *Bifidobacterium* strains tested had lower degradation activity than the lactobacilli, possibly due to intrinsic oxalate toxicity toward the former genus.

In the search for efficient oxalate degraders, laboratories have also screened novel isolates from the guts of various animals. Murphy et al. [38] isolated *Bifidobacterium* and *Lactobacillus* strains from cats and dogs, and demonstrated *in vitro* oxalate degradation in 61 % of the *Lactobacillus* isolates. In contrast, the *Bifidobacterium* spp. showed very little degradation activity for all species tested. Two strains of *L. murinus* and two of *L. animalis* were tested in a rat model, but only the *L. animalis* strains gave significant reduction in urinary oxalate levels. Other workers have screened probiotic strains for both oxalate-degradation activity and also their ability to modulate inflammation in the human GIT, and have found strains of *L. plantarum*, *L. acidophilus*, *B. breve* and *B. longum* to be promising candidates for this purpose in *in vitro* testing [17].

Gut Colonisation and *In Vivo* Bacterial Oxalate Utilization Studies in Humans

A number of human *in vivo* studies have been undertaken to examine the effect of oral administration of lactic acid bacteria probiotics on dietary hyperoxaluria, plasma oxalate concentration and urinary oxalate excretion. However, most of these studies are limited in various ways, with low numbers of participants and varying test procedures making evaluation of their comparative effectiveness difficult.

The earliest studies suggested that lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium* in particular, reduced urinary oxalate excretion by 40–50 % in a study concerning six calcium-stone forming participants [11]. The reduction persisted after the treatment ceased. This was followed by a study with participants with enteric hyperoxaluria, where the Oxadrop (VSL Pharmaceuticals) probiotic preparation was used. This was comprised of a mixture of four lactic acid bacterium species (*L. acidophilus*, *L. brevis*, *S. thermophilus* and *B. infantis*). One packet of Oxadrop per day reduced urinary oxalate excretion by 19 %, and this increased to 24 % when two packets per day were administered [28]. This study had 20 participants, 10 receiving Oxadrop daily and 10 in the placebo group.

However, more recent studies, using randomized double-blind groups of stone-formers with idiopathic hyperoxaluria, were unable to show a reduction in urinary oxalate excretion, despite using the same Oxadrop probiotic preparation at the same concentration of $4–9 \times 10^{11}$ cfu. The sample size in each case was also a total of 20 participants, 10 receiving Oxadrop daily and 10 in the placebo group [18]. Lieske et al. [29] conducted a double-blind study to determine the effect of Oxadrop and the synbiotic AKSB preparation containing fructooligosaccharides, *Enterococcus faecium* and *Saccharomyces cerevisiae* (Agri-King Synbiotic, Fulton, USA) on 40 participants with mild hyperoxaluria receiving an oxalate controlled diet. They concluded that while the restricted diet reduced urinary oxalate, the probiotics and synbiotics did not. However, it is not clear what the basis of the choice of the organisms in the AKSB preparation was, nor how they might influence oxalate metabolism. Recently, Siener et al. [49] placed 20 healthy participants on an oxalate-rich diet for 6 weeks, and administered Oxadrop for 5 weeks. Urinary oxalate excretion and plasma oxalate concentrations increased with the oxalate-rich diet, and were not reduced by the ingestion of probiotics in the treated group.

A different probiotic preparation, VSL#3 (Sigma Tau Pharmaceuticals, USA) was used in two studies. This formulation contained eight different *Lactobacillus*, *Streptococcus* and *Bifidobacterium* strains, none of them the same as Oxadrop. In the first of these studies, a significant reduction in urinary oxalate excretion was observed in subjects with an oxalate-rich diet after probiotic administration, but this occurred only in those four subjects with a very high level of oxalate absorption from the gut [39]. In the second study, 11 healthy participants were given a high oxalate diet of 176 mg oxalate per day, and either one dose or two doses of VSL#3 daily. The results showed that either dosage of probiotics reduced urinary oxalate and increased oxalate absorption. However, the absorption was only monitored 6 h after ingestion, and not after a 24-h period as in other studies [4]. Some laboratories isolate and test their own probiotic mixtures. Ferraz et al. [16] treated 16 stone-forming patients with hyperoxaluria while on a controlled oxalate-rich diet with a mixture of *L. casei* and *B. breve,* but had variable results in the lowering of urinary oxalate.

Liebman and Al-Wahsh [27], in reviewing the effects of probiotics on dietary oxalate absorption, concluded that the variation in the results between laboratories was due to differences in protocols, variations in diet and perhaps the choice and administration of the specific probiotics. However, there is evidence that probiotics can, in certain cases, contribute to a reduction in oxalate after a high oxalate diet

[39] or in cases of enteric hyperoxaluria [28]. There is, therefore, a need for more rigorous studies on the use of oxalate-degrading lactic acid bacteria for the treatment of patients at risk of developing kidney stones. Effective bacterial strains and administration protocols have still to be developed and proven for future therapeutic applications.

In particular, it will be important to consider the issues around the benefits of chronic administration of the oxalate degrading probiotics as opposed to undertaking permanent recolonisation of the guts of patients suffering from enteric hyperoxaluria. There is currently limited information correlating the lack of a particular oxalate-degrading bacterial population and the development of kidney stone disease due to the variations in normal base-line microbial numbers [23]. The many factors affecting microbial colonisation, including diet and host-microbial interactions, also require further investigation [41, 56].

Novel Recombinant Approaches (Oxalate Decarboxylase from *B. subtilis* and Expression in *L. plantarum*)

A more recent alternative approach to enhancing microbial oxalate degradation in the gut is to introduce higher levels of an oxalate degrading enzyme, such as oxalate decarboxylase, either delivered in the form of *in vitro* purified recombinant enzyme or expressed *in vivo* in a naturally occurring recombinant LAB species which was previously unable to degrade oxalate [47]. Oxalate decarboxylase (OxDC) (EC4.1.1.2) has been found in several fungi [51] as well as certain bacteria, such as *Bacillus subtilis* [52], and catalyses the conversion of oxalate to carbon dioxide and formate [22]. The *B. subtilis* OxDC enzyme is encoded by the *oxdC* gene (formerly known as *yvrk*) and was induced under acidic conditions (pH 5.0) but not by the presence of oxalate. The purified *B. subtilis* OxDC activity was catalytically dependent on low concentrations of O_2 [42]. However, it retained up to 76 % of its activity when tested *in vitro* under anaerobic conditions at pH 5.0 [52]. In addition, since the human gut demonstrates an intraluminal oxygen gradient [3], there is the potential availability of catalytic concentrations of oxygen sufficient to drive the enzyme reaction *in vivo*. These features make the OxDC enzyme an appealing candidate for possible heterologous expression and use in the treatment of hyperoxaluria.

Use of Free Oxalate Decarboxylase for *In Vivo* Oxalate Degradation

The first work reported in this field aimed at introducing oxalate decarboxylase into the gut through the direct ingestion of *in vitro* purified recombinant protein. The perceived advantage of this approach was that it aimed at providing a specific dose of a stable and active oxalate degrading enzyme throughout the gut [19].

The *B. subtilis* OxDC enzyme was expressed in *E. coli* and purified [22]. The processed protein crystals were cross-linked with gluteraldehyde (OxDC-CLEC^{R}) to provide an active preparation for administration in *in vivo* trials using mice suffering from hyperoxaluria due to an *Agxt* gene (alanine-glyoxylate aminotransferase) mutation [19]. It was found that a daily dose of 200 mg of the preparation, given for 16 days, reduced the faecal oxalate concentration of the mice by 72 %, and the urinary oxalate by 44 %, relative to a control group receiving a placebo (n = 7 per group). When the experiment was repeated in the presence of ethylene glycol (EG), which induced nephrocalcinosis and kidney failure, it was found that 80 mg per day of the OxDC-CLECR preparation given to the test group (n = 11) for 32 days was sufficient to cause a urinary oxalate reduction of 40 %, prevented the EG-induced kidney pathology, and there was 100 % animal survival.

Cowley et al. [13] developed an improved recombinant mutant form of the *B. subtilis* OxDC enzyme by replacing the cysteine residue at position 383 with a serine to prevent auto-aggregation of the protein without loss of enzyme function *in vitro*. This compound was named OC4 (Oxazyme). Its oral toxicity in rats and dogs was evaluated by oral gavage over a 14 day period at 50 times the anticipated clinical dose, and no adverse toxic or adverse effects were observed during this period or in a subsequent 7 day recovery period. The oxalate degrading ability of Oxazyme was also tested *in vitro* in simulated gastric and intestinal environments [37]. The oxalate decarboxylase enzyme completely degraded oxalate derived from potassium oxalate under pH conditions equivalent to those found in the stomach and the proximal colon. It also significantly reduced the oxalate concentration of homogenised spinach (a food with a high oxalate content) under both conditions. Oxazyme was subsequently used in a limited non-randomised human clinical trial at the Mayo Clinic (ClinicalTrials.gov Identifier: NCT01127087; Principal Investigator Dr John Lieske, MD). All participants showed no adverse effects, and there was some indication of a statistically significant reduction in urinary oxalate. However, the number of participants completing the trial was very small, and the need for a more robust, extensive study is indicated.

Expression of Oxalate Decarboxylase in a Suitable Heterologous Probiotic Host Bacterium

Oxalate decarboxylase could also be delivered to the gut as a recombinant enzyme expressed *in vivo* in a suitable LAB species with the aim of enhancing oxalate degradation in the human gut and the prevention of hyperoxaluria. One of the major criteria that need to be considered when selecting a suitable heterologous host probiotic strain for human use is that the bacterium should effectively colonise the mucosal surface of the gut. Studies on *Lactobacillus plantarum* adhesion to the gut extracellular matrix (ECM) showed that four of the 16 indigenous *Lb. plantarum* strains tested showed significant ability to bind to both the fibronectin and mucin

ECM components [58]. This organism, therefore, shows potential as a delivery vehicle for the OxDC enzyme as well as the potential capacity for long-term colonisation of the gut.

Cloning of *oxdC* from *B. subtilis* and Heterologous Expression in *Lb. plantarum*

Kolandaswamy et al. [24] examined the over-expression of the *B. subtilis oxdC* gene in *Lb. plantarum* NC8, a GRAS bacterium which does not normally express oxalate decarboxylase. The gene was cloned onto a shuttle vector and its expression was regulated from an inducible promoter by sakacin-P using the pSIP high-level expression system [50] thus producing an active recombinant OxDC protein. However, the expression of this enzyme in *Lb. plantarum* was intracellular and it was generated at very low levels from the inducible promoter to which the gene was fused.

With a view to improving these shortcomings and developing a recombinant strain which might degrade oxalate extracellularly and more efficiently and under human gut conditions, Sasikumar et al. [47] investigated the use of signal peptides to drive the secretion of the recombinant enzyme. *Lb. plantarum* WCFS1, a human saliva isolate, was used to express the *B. subtilis oxdC* gene cloned downstream of the same sakacin-P (inducible) promoter used by Kolandaswamy et al. [24] but this time fused to homologous peptide sequences Lp_0373 or Lp_3050, which had previously been shown to enhance secretion in this host [34, 35]. They found that a functional OxdC protein was secreted efficiently from cells carrying these constructs and could degrade up to 50 % of the oxalate in the growth medium.

Anbazhagan et al. [6] further developed the system by introducing a constitutive promoter, which required no induction, ahead of the signal sequences and the cloned *oxdC* gene. The *Lb. plantarum* L-lactate dehydrogenase promoter was successfully used to overexpress functional, extracellular oxalate decarboxylase, suggesting that, in this form, the organism expressing the recombinant protein might be useful for degrading dietary oxalate in the gut.

In Vivo Evaluation of Recombinant *Lb. plantarum* Expressing OxDC

In a recent study, Sasikumar et al. [45, 46] compared the *in vitro* and *in vivo* oxalate degrading ability of *Lb. plantarum* WCFS1, expressing OxDC constitutively either intracellularly or extracellularly *in vitro*, in male wistar albino rats. All rats (n=30) received 5 % potassium oxalate in their normal diets but the test groups (n=5 per group) were, in addition, fed either of the two forms of the recombinant *Lb. plantarum* daily

from day 14 for an additional 2 weeks. It was found that the rats receiving the recombinant bacterium showed a significant reduction in urinary oxalate as well as calcium, creatinine and uric acid as compared to the control group, and that the excreted form of the enzyme achieved this more efficiently. Examination of the kidney homogenates of the various groups also showed significantly reduced oxalate levels in the test group, and there was no microscopic histological sign of calcium oxalate crystallisation when compared to the rats which had not ingested the recombinant bacteria. The authors conclude that daily oral administration of a biologically contained bacterium expressing the enzyme in a secreted form may be a useful therapy for the treatment of calcium oxalate kidney stone disease.

Conclusions

The use of LAB as probiotics for the control of kidney stone disease remains a potentially useful therapy. However, since the capacity of the bacteria to degrade oxalate is highly species and strain specific, it is not yet clear whether organisms specifically selected on the basis of *in vitro* oxalate degradation capacity perform in the same way in the gut. The current results of certain *in vivo* trials seem promising, however, far more rigorous double-blind placebo controlled trials need to be conducted to verify the efficacy of this therapeutic intervention.

Overall, direct supplementation of purified oxalate decarboxylase enzyme to patients with oxaluria seems a most promising clinical approach, however, this also needs to be more rigorously tested under *in vivo* conditions. Delivery of the enzyme via extracellular expression in a suitable heterologous host, such as *Lb. plantarum,* is also a worthwhile direction for future research. In particular, the colonisation ability of the plasmid containing host strain, and the enzyme expression and activity levels achieved *in vivo,* need to be examined further.

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Chapter 8
Role of *Oxalobacter formigenes* Colonization in Calcium Oxalate Kidney Stone Disease

John Knight and Ross P. Holmes

Abstract *Oxalobacter formigenes* is part of the bacterial flora in the large intestine of humans and many other mammalian species. It is unique in that it requires oxalate both as an energy and carbon source. A lack of colonization with *O. formigenes* is a risk factor for idiopathic recurrent calcium oxalate stone disease. Protection against calcium oxalate stone disease appears to be due to the oxalate degradation that occurs in the gut as measurements of 24 h urinary oxalate indicate that *O. formigenes* colonized calcium oxalate stone formers excrete less oxalate compared to non-colonized individuals when ingesting standardized diets. There is also some evidence that suggests a possible mechanism involving intestinal oxalate secretion triggered by the bacterium itself, as *O. formigenes* colonization appears to lower plasma oxalate. Whether high oral doses of this organism can promote sufficient intestinal oxalate secretion to diminish the oxalate burden on the kidney in individuals with Primary Hyperoxaluria is currently being tested by OxThera, Inc. in a phase 2 clinical trial. Much still remains to be learned about how *O. formigenes* establishes and maintains gut colonization and the precise mechanisms by which it modifies stone risk.

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 [2, 4]. 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

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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 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 [23]. For example, 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 [23]. For example, the OXCC13 genome contains genes that code for four proteins that are homologous to the type 1 pilus proteins [7], and appear to be in an operon. However, the draft sequence of HOxBLS does not harbor genes that show good homology to these OXCC13 pilus proteins. Pili facilitate cell-to-cell transfer of genetic material, adhesion to host cells and the transfer of molecules to host cells, suggesting these functions in the two sequenced strains are different.

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 [9, 10]. 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. In particular, studies that examine the viability of *O. formigenes* following typical probiotic preparative procedures such as freezing and lyophilization warrant further investigation.

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 [5, 24].

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 recent 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. 8.1 [19]. In this study, bacterial numbers were shown to

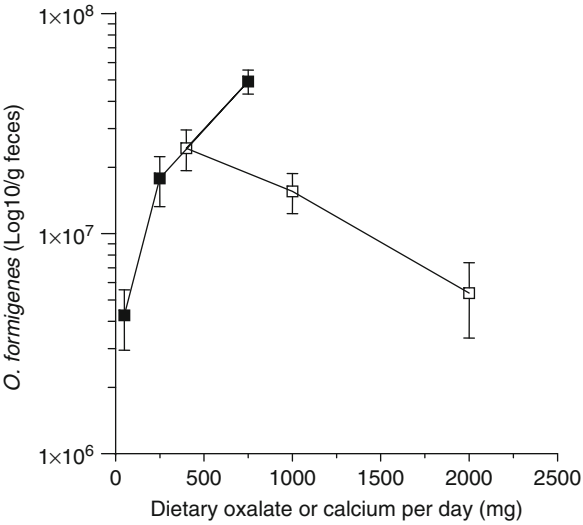

Fig. 8.1 Number of fecal *O. formigenes* with changes in dietary oxalate (■) or dietary calcium (□). Daily calcium intake was 1000 mg on the varied oxalate dietary phase and daily oxalate was 250 mg on the varied calcium dietary phase. Real-time PCR was used to quantitate *O. formigenes* numbers. 5.5×10^4 CFU/ng DNA was used to convert qPCR data to number of *O. formigenes* per g feces (Modified from Jiang et al. [19]. With permission from Elsevier)

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 its availability 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 stool suggests *O. formigenes* represents a tiny fraction of the total intestinal microbiota [23]. Many low abundance bacteria are thought to survive in the intestines by occupying specific nutrient niches where competition for their food source is limited [12]. Indeed, both *in vitro* culture studies [29] and a recent human study [19] 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.

In a recent study, total stool oxalate measurements showed *O. formigenes* colonized individuals excrete significantly less oxalate than non-colonized individuals when consuming diets controlled in their level of nutrients including oxalate and calcium [19] (Fig. 8.2), 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. The impact of these "generalist" oxalate degrading bacteria in calcium oxalate stone disease is not known. Several human studies that have assessed the ability of bacteria with oxalate degrading potential to reduce urinary oxalate excretion have led to promising but

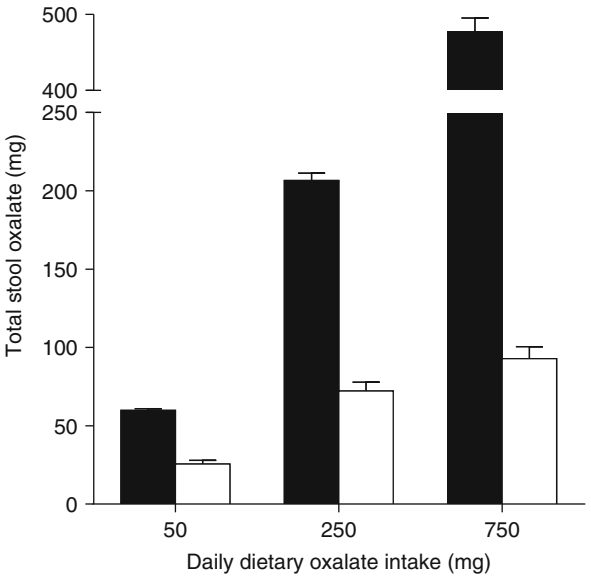

Fig. 8.2 Stool oxalate of *O. formigenes* colonized (□) and non-colonized healthy subjects (■) on nutrient controlled diets varying in oxalate. Daily calcium intake was 1000 mg (Modified from Jiang et al. [19]. With permission from Elsevier)

generally mixed results. For example, the intake of commercial probiotic preparations, including Oxadrop® (VSL Pharmaceuticals), that contain a mixture of lactic acid bacteria known to degrade oxalate *in vitro*, reduced urinary oxalate excretion in patients with enteric hyperoxaluria [26] or after an oral load of oxalate [1]. However, when tested in healthy subjects consuming an oxalate rich diet [33], in patients with idiopathic hyperoxaluria [13], or patients with mild hyperoxaluria [27] probiotic supplementation did not reduce urinary oxalate significantly, suggesting chronic supplementation with "generalist" oxalate degrading bacteria may only be beneficial for patients with absorptive (enteric) hyperoxaluria.

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 [32] and based on what we know about *O. formigenes* transmission from animal experiments it is obtained from the environment, not directly from the mother [8].

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, 21]. Several studies have indicated that the intake of antibiotics can result in the loss of colonization [21, 22, 25, 28], and this is supported by lower prevalence of *O. formigenes* in both cystic fibrosis patients [30], and calcium oxalate stone formers

who are frequently prescribed antibiotics [28, 31]. 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 oxalate intake. The impact of dietary oxalate deprivation on *O. formigenes* colonization warrants further examination.

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* [11], 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 [17, 18]. 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 [6, 28, 35], as measurements of urinary oxalate excretion were 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 [34]. 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 [19]. In this study, the most beneficial effect of *O. formigenes* was observed when colonized

subjects were administered a low calcium (400 mg/day) and moderate oxalate (250 mg/day) diet, urinary oxalate excretion was found to be lowest 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*.

Interestingly *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 [14–16]. 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) [34]. 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 2 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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Chapter 9
BCG for the Treatment of Non-muscle Invasive Bladder Cancer

Roland Seiler and Peter C. Black

Abstract Bacillus Calmette Guérin (BCG) was developed primarily as a vaccine against tuberculosis, but was found early on to demonstrate an antineoplastic effect of in different cancers. Today the administration of BCG in oncology is limited to intravesical instillation in non-muscle invasive bladder cancer (NMIBC). Although BCG is one of the most investigated medical cancer treatment in urology, its mechanisms of action are still not fully understood. After instillation into the bladder and internalization of BCG in bladder cancer cells and macrophages, BCG triggers apoptosis of neoplastic cells and promotes activation of T-cells that are responsible for the long-term antitumor defence. BCG is indicated as adjuvant treatment of most patients with high risk NMIBC, and subsequent maintenance treatment for up to 3 years can reduce recurrence and progression. Despite this reduction and optimal BCG therapy, careful patient follow-up is required to detect bladder cancer progression. Ongoing studies are investigating different agents for co-treatment with BCG or modulation of BCG, in order to increase the efficacy of BCG and improve patient outcomes.

Background

The History of BCG

BCG was developed for 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 transfered to Albert Calmett and Camille Guérin in the Pasteur Institute in Lille. After 231 passages (from 1908 to 1921), the initial

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extremely virulent strain became avirulent. In 1921 the first BCG vaccination was successfully given to a newborn girl. After dissemination of this vaccination, the mortality of 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 by BCG. It was not until after this application in patients that the first landmarks in animal models were achieved 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 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 of the bladder 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 treatment compared to the period before treatment in 9 patients with NMIBC. This regimen was subsequently validated at the University of Texas in San Antonio and at Memorial Sloan-Kettering Cancer Center (MSKCC) 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. The pivotal step in BCG

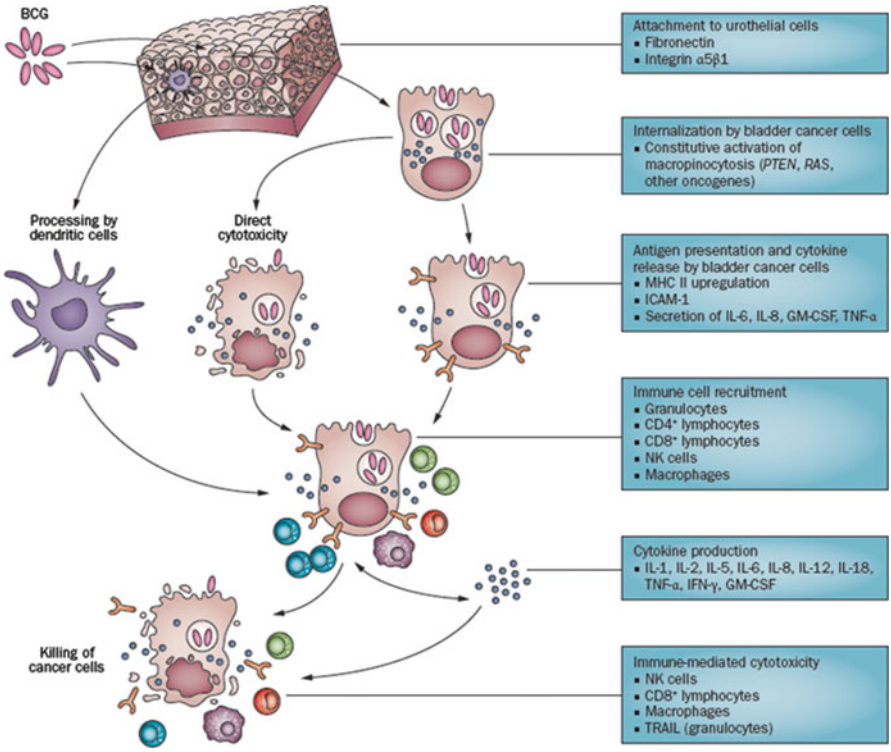

Fig. 9.1 Mechanism of action of BCG in bladder cancer [33]

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 histocompatibility complex and ICAM-1) is modified. This antigen presentation to CD4+ T cells results in cytokine release (Th1 cytokine profile: NF-y, IL-2, IL-12 and TNF-α) and triggers apoptosis of neoplastic cells by natural killer cells, cytotoxic CD8+ cells and neutrophils (Fig. 9.1). Neutrophils are also responsible for BCG treatment-related secretion of TNF-related apoptosis-inducing ligand (TRAIL), a 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 [7]. 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 [8].

Intravesical BCG: Current Guidelines and Controversies

Indication

Intravescial 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). Intermediate risk disease is defined as low grade tumor that is either greater than 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 continues to be used fairly universally [4]. Most of the controversy regarding the therapeutic regimen relates to maintenance therapy. Despite anecdotal claims to the contrary [9], the evidence is clear that maintenance therapy reduces the risk of recurrence and progression [10]. The best data supports the use of the full Southwest Oncology Group (SWOG) 8507 regimen, with 3 weekly doses of BCG at 3, 6, 12, 18, 24, 30 and 36 months after induction [5, 11]. The more recent European Organization of Research and Treatment of Cancer (EORTC) 30962 trial comparing 1 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 were sequenced completely and differences in the genetic variability have 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, efficacy for inducing tumor growth inhibition and triggering cytokine production [12]. The Connaught and Tice strains are the most widely administered in North America and Europe. Although they 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 recent 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 %) [13]. The efficacy of BCG Tice was also inferior to BCG RIVM in an older study [14] (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.

Another critical unmet need in this context is a biomarker to guide patient selection for intravesical BCG and maintenance therapy. Various biomarkers (e.g. gene signatures, methylation status, interleukin-2 gene expression, urinary interleukin-8 and 18) have been investigated in order to predict efficacy of BCG treatment [15–18]. One with potential clinical utility is multi-color fluorescent in situ hybridization (FISH) [19], 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. 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 [11, 20]. Compared to 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 seems 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 9.1). Serious side effects are encountered in <5 % of patients and can be treated effectively in almost all cases [21]. 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 of BCG administration. The recent EORTC trial

Table 9.1 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. [21], Witjes et al. [22], Gontero et al. [23]
[a]Including fluoroquinolone/isoniazid/rifampin

demonstrated that dose reduction to 1/3 dose BCG had no benefit on toxicity, so that this measure should be avoided [6]. The concomitant use of antibiotics can reduce toxicity [24], but longer follow-up and larger patient cohorts would be necessary to demonstrate that there is no detrimental effect on disease outcome [25].

BCG Failure

Several meta-analysis 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) [20].

Table 9.2 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)

Nieder et al. [26]

Several common definitions of BCG failure are defined in Table 9.2 [26, 27]. The management of patients who have failed BCG therapy is complex. The risk of pogression increases with each subsequent course of intravesical therapy, and no therapies have more than an approximate 20 % response rate at 1 year. 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 [28].

If bladder preservation is sought in BCG resistant and refractory disease, several strategies are now available [29]. BCG can be combined with interferon-alpha, although there is no evidence that this is any more efficacious than repeat BCG along. Small phase II trials suggest that intravesical gemcitabine and docetaxol are well-tolerated alternatives for BCG failure with measurable but modest activity. Electromotive delivery of mitomycin alternating with BCG over 12 months proved superior to a similar, non-standard course of BCG alone in a prospective randomized clinical trial involving patients with high-risk NMIBC but not prior intravesical BCG. This method is pending evaluation in a U.S. clinical trial prior to being approved by the FDA. 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 9.3. 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 9.3 Clinical trials registered on Clincal.Trials.gov involving intravescial BCG therapy for bladder cancer (accessed Oct. 10, 2014)

NCT number	Title	Experimental agent	Design	Outcome measures	Completion date
	BCG combination trials				
NCT02010203	A phase 1/2 study of HS-410 in patients with non-muscle invasive bladder cancer after TURBT	HS-410: tumor vaccine	Randomized, phase 1/phase 2	Phase 1: safety and tolerability Phase 2: 1-year disease-free survival	May 2017
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; stimulates CD8+ T- and NK-cells	Non-randomized phase 1/phase 2	Safety and tolerability overall survival	October 2018
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	Disease-free survival (36 months)	December 2019

NCT01878188	Pilot study of BC-819/PEI and BCG in patients with superficial transitional cell bladder carcinoma	BC-819/PEI: double stranded DNA plasmid, that carries the gene for the diphtheria toxin A	Non-randomized phase 1	Safety and tolerability	September 2014
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 in patient with high risk non-invasive urothelial carcinoma. Toxicity related to treatment with BCG followed by sunitinib	June 2013
	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	PANVAC (poxviral-based vaccine therapy targeting CEA and MUC1 in carcinoma)	Randomized, phase 2	Improvement in disease-free survival	November 2017
NCT01259063	RAD001 and intravesical gemcitabine in BCG-refractory primary or secondary carcinoma in situ of the bladder	RAD001 (Everolimus): mTOR inhibitor	Non-randomized, phase 1/phase 2	Phase I – to establish the dose-limiting toxicity Phase II – to determine disease free rate at 1 year	December 2014

(continued)

Table 9.3 (continued)

NCT number	Title	Experimental agent	Design	Outcome measures	Completion date
NCT01732107	Dovitinib in BCG refractory urothelial carcinoma with FGFR3 mutations or over-expression	Dovitinib: multi-tyrosine kinase inhibitor (especially FGFR and VEGFR)	Phase 2	Complete response rate/ disease-free survival rate Rate of progression/partial response rates/treatment-related toxicity	March 2016
NCT01625260	A study of ALT-801 in patients with Bacillus Calmette-Guerin (BCG) failure non-muscle invasive bladder cancer	ALT-801: recombinant protein consisting of interleukin-2 fused to a humanized soluble T-cell receptor directed against a p53-derived antigen	Phase 1/phase 2	Safety profile/tolerability	July 2014
NCT02009332	Phase 1/2 study of ABI-009 in BCG refractory or recurrent nonmuscle invasive bladder cancer	ABI-009 (nab-rapamycin): nanoparticle albumin-bound rapamycin, mTOR inhibitor	Phase 1/phase 2	Adverse events as a measure of safety and tolerability; time frame: end of study 3 months and follow-up 1 year	December 2015

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 [30]. 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 [31]. A new trial testing BCG vaccination in patients planned for intravesical BCG is under development by SWOG ("Prime Trial"). A related trial in Europe, where patients are much more likely to have been previously exposed to BCG vaccination, is in the concept phase ("Boost Trial").

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. In addition, several antigens that are exclusively expressed in various tumor types have been discovered recently. This raises the hypothesis that modified BCG strains that carry representative 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 [32]. However, these options have only been investigated in *in vitro* and *in vivo* models without clinical experience.

Conclusions

Although introduced more than 30 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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