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The human urobiome

L. Brubaker[1] · C. Putonti[2,3] · Q. Dong[4] · A. J. Wolfe[5]

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Abstract

Traditionally, the healthy urinary bladder has been considered to be sterile. Several teams have used metagenomic (DNA-dependent) and metaculturomic (culture-dependent) methods to debunk this longstanding dogma. In fact, resident microbial communities (urobiome) have been detected in both adult females and males. Although the field is young, several observations have been made. For example, the urobiome differs between men and women, likely due to anatomical and hormonal differences. Importantly, the urobiome has been associated with a variety of lower urinary tract disorders, including overactive bladder and post-operative urinary tract infection, raising the possibility that clinicians might one day treat symptoms by modifying the urobiome instead of killing the suspected uropathogen. Little is known concerning the relationship between the urobiome and host genetics; so far, only a single paper has reported such a study. However, major efforts have gone into understanding the genomics of the urobiome itself, a process facilitated by the fact that many urobiome studies have used metaculturomic methods to detect and identify microbes. In this narrative review, we will introduce the urobiome with separate sections on the female and male urobiomes, discuss challenges specific to the urobiome, describe newly discovered associations between the urobiome and lower urinary tract symptoms, and highlight the one study that has attempted to relate host genetics and the urobiome. We will finish with a section on how metagenomic surveys and whole genome sequencing of bacterial isolates are improving our understanding of the urobiome and its relationship to lower urinary tract health and disorders.

Introduction

Microbiome research is advancing rapidly. For example, until a decade ago, the human urinary tract was considered a sterile system. However, this is not true, as researchers have used metagenomics (DNA-dependent) and metaculturomics (culture-dependent) technologies to discover and confirm the existence of microbes (microbiota) in the human urinary tract and to characterize them and their genomes (microbiome) (Wolfe et al. 2012; Price et al. 2016; Khasriya et al. 2013; Hilt et al. 2014; Fouts et al. 2012). For reviews, see (Whiteside et al. 2015; Price et al. 2017; Mueller et al. 2017; Brubaker and Wolfe 2016).

The umbrella term "urobiome" broadly refers to both the microbiota and microbiomes of the urinary tract (Wolfe and Brubaker 2018). As with other human microbiota/microbiomes, there is much to learn about the urobiome, including the mechanisms that govern establishment, regulation, disruption and resilience of this microbial community in healthy states. Early urobiome research provides evidence for associations between the urobiome and common urinary disorders and symptom states, although mechanistic research is needed.

Human microbiota can be composed of bacteria, archaea, viruses, and eukaryotic microbes such as fungi (Lloyd-Price et al. 2016). In each niche, certain communities are associated with preferred—"healthy"—biological states. This is also true of the urobiome, although the preferred urobiome states differ by gender and appear to change with age (Wolfe

✉ A. J. Wolfe
awolfe@luc.edu

1 Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Diego, La Jolla, CA, USA

2 Bioinformatics Program, Loyola University Chicago, Chicago, IL, USA

3 Department of Biology, Loyola University Chicago, Chicago, IL, USA

4 Department of Medicine and Center for Biomedical Informatics, Stritch School of Medicine, Loyola University Chicago, Maywood, IL, USA

5 Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, IL 60089, USA

and Brubaker 2018; Price et al. 2019). The metabolic products of microbiota are central to community function and regulation, and influence the niche in which they live (Xu et al. 2014; Flint et al. 2012). Currently, little is known of the metabolites of individual microbes of the urobiome or of the whole community. Finally, viruses that exclusively infect bacterial cells (bacteriophages, phages) are present in the urobiome, vastly outnumbering eukaryotic viruses (Garretto et al. 2019). The role of these phages in health and disease is largely unknown.

Female urobiome

In studies of urine samples obtained by transurethral catheterization of adult women without urinary symptoms, it is clear that multiple urinary community state structures, called urotypes, are associated with urinary health (Pearce et al. 2014). The most common "healthy" urotypes are predominated by the same species of the genus *Lactobacillus* that inhabit the vagina (Price et al. 2019; Pearce et al. 2014). Whether the lactobacilli that inhabit the female urinary and genital tracts are of the same lineage remains to be seen, but some evidence supports that conclusion (Thomas-White et al. 2018a). Other common urobiomes are predominated by species of the genera *Gardnerella, Streptococcus, Staphylococcus, Corynebacterium* and *Escherichia*. Some evidence suggests that the female urobiome can change with age; for example, the urotype predominated by *Gardnerella* is more common in pre-menopausal women, whereas the urotype predominated by *Escherichia* is more common in post-menopausal women (Price et al. 2019). Limited longitudinal evidence reveals the ability of an individual adult woman's urobiome to change in composition over days, while simultaneously demonstrating longer-term stability. A common theme is alternation between a community predominated by a *Lactobacillus* species with a community predominated by a *Gardnerella* species. One intrinsic factor (menstruation) and one extrinsic factor (vaginal sex) have been associated with disruption of this pattern; however, the urobiome appears to be resilient, returning to the normal pattern within a few days (Price et al. 2020b).

The urobiome of adult women has been associated with vulnerability to UTI, especially at high-risk times, including catheterization or other urinary tract instrumentation at the time of pelvic surgery (Thomas-White et al. 2018b; Fok et al. 2018). Other life events (e.g., sex and pregnancy) with elevated prevalence of UTI may also be associated with the urobiome. Risk-stratification using urobiome characteristics may allow targeted prevention research and improved clinical care. In addition, the urobiome of adult women has been associated with common forms of urinary incontinence, including both urgency urinary incontinence and stress urinary incontinence (Pearce et al. 2015, 2014; Price et al. 2020a; Karstens et al. 2016). Further research will be needed to explain the mechanisms underlying the detected differences between incontinent women and continent controls. Within the group of women accepting oral medication (solifenacin) for treatment of urgency urinary incontinence, the pre-treatment urobiome appears to be associated with treatment response. Women whose symptoms improve tend to have urobiomes with characteristics that resemble those of unaffected women. These urobiomes were simple with few species and a predominant taxon, most often a *Lactobacillus* species. In contrast, women who did not respond to treatment tended to have more complex urobiomes with no predominant species (Thomas-White et al. 2016a). In addition, treatment response may be associated with urobiome resilience and restoration. Additional studies are necessary to understand the relationship between urobiome characteristics and treatment response. Such studies should help clinicians to provide personalized treatment to improve patient outcomes.

Male urobiome

Most of the early urobiome work focused on adult women; however, the first microbiome studies were performed on the male urethra. These early studies provided evidence for a voided urinary microbiome that closely resembled the distal urethra (Dong et al. 2011), that the microbiome was different in in men with and without sexually transmitted infections (Nelson et al. 2010), and that the urethral microbiome may be influenced by partnered sexual activity (Dong et al. 2011; Nelson et al. 2010). More recent research has provided evidence that the urobiome is relevant to lower urinary tract symptoms in men. Benign prostatic hyperplasia, a common finding in older men, is frequently associated with urinary symptoms such as urinary frequency and urgency. Several small studies suggest that the urobiome detected in catheterized urine samples differ between affected and unaffected men, with a higher level of symptoms associated with bacterial load (Bajic et al. 2020). Urobiome research is raising hope for men who suffer with chronic prostatitis and/or chronic pelvic pain syndrome; these distressing conditions have been poorly understood and ineffectively treated. However, differences in bacterial composition and species diversity may lead investigators to new insights and more effective therapeutics (Nickel et al. 2015; Mändar et al. 2017; Shoskes et al. 2016).

Urobiome and cancer

It is well known that certain microbes are highly associated with, and in some cases etiologic agents for, human cancer. This also may be true for the urobiome and a few

studies have attempted to find differences between individuals with and without either prostate or bladder cancer (Popović et al. 2018). Although this is an important line of investigation, the sample sizes are too small to come to any strong conclusions (Popović et al. 2018; Shrestha et al. 2018; Wu et al. 2018). If the urobiome does relate to urinary tract-associated cancer, characteristics of the urobiome may be useful as a noninvasive biomarker. More exciting is the potential modifiability of the urinary bacterial community, especially given the use of immunotherapies, such as intravesical bacillus Calmette-Guérin (BCG), a main treatment for high grade non-muscle-invasive urothelial cell cancer (Bajic et al. 2019). The activity of BCG relies on binding to urothelial fibronectin. Interestingly, *Lactobacillus iners* appears to be superior at binding fibronectin (McMillan et al. 2013). Clearly there is potential for the urobiome to modulate BCG response, highlighting the importance of further study into the relationship between bladder cancer and the urinary microbial community.

The urobiome may have a role in more advanced and metastatic disease when other immunotherapy agents are increasingly utilized. Immunotherapies that rely on the PD-1/PD-L1 axis have been studied in the other human microbial niches. The efficacy of anti-PD-1 therapy for treatment of metastatic melanoma may be related to the presence of specific microbes (the species *Bifidobacterium longum*, *Collinsella aerofaciens* and *Enterococcus faecium*) (Matson et al. 2018).

Urobiome-specific challenges

Urobiome research poses challenges, unlike certain other human microbiomes, such as the gut. First, the urobiome is a very low biomass microbial niche with a high risk of specimen contamination. Thus, collection methods are especially critical (Wolfe et al. 2012; Southworth et al. 2019; Chen et al. 2020). Second, because of anatomy and hormones, there are substantial gender differences (Abelson et al. 2018). Third, low biomass samples are exquisitely sensitive to contaminants, from the environment and from the reagents used to prepare samples for sequencing (Stinson, Keelan, and Payne 2019; Olomu et al. 2020).

Early research used suprapubic aspirates (with control suprapubic needle passage) in adult women to confirm the presence of microbes in the human bladder. Urine specimens collected using transurethral catheterization reasonably approximated suprapubic specimens. Too often neither resembled midstream voided specimens. Since suprapubic aspiration bypasses vulvovaginal contamination and because catheterization is less invasive, catheterization is the preferred method of collection for research purposes (Wolfe et al. 2012). This method has been used in both women and men to characterize the bladder urobiome (Pearce et al. 2014, 2015; Thomas-White et al. 2016a; Fok et al. 2018; Thomas-White et al. 2018b; Price et al. 2019; Bajic et al. 2020; Karstens et al. 2016; Ackerman and Underhill 2017; Adebayo et al. 2020; Fouts et al. 2012). In men and women, the urethral microbiome, as determined by the use of swabs, appears to be distinct from the bladder microbiome (Bajic et al. 2020; Chen et al. 2020). Voided urine samples, a mainstay of clinical evaluation, too often contain microbes that inhabit the vulvo-vaginal tissues (Wolfe et al. 2012; Southworth et al. 2019; Chen et al. 2020). The ease of specimen collection using voided collection allows larger populations to be studied without undergoing urethral catheterization; however, interpretation of voided specimens must consider the knowledge that the detected microbes are not all urobiome members and, in many cases, the majority of the microbes are genital in origin. Thus, in women, voided specimens reflect the urogenital microbiome. In men, however, voided urine often reflects the urethral microbiota (Dong et al. 2011; Chen et al. 2020).

As stated earlier, the most common urotype in adult women without symptoms is predominated by *Lactobacillus* (Pearce et al. 2014, 2015; Komesu et al. 2018; Price et al. 2019). In contrast, *Streptococcus* and *Staphylococcus* appear to predominate in men without lower urinary tract symptoms, but again the sample sizes are still quite small (Bajic et al. 2020; Shrestha et al. 2018; Frølund et al. 2018; Dong et al. 2011; Nelson et al. 2010). The reason why *Lactobacillus* is a common urobiome member of women, but not men, is almost certainly the result of hormonal differences. For example, estrogen enhances the production of glycogen, which *Lactobacillus* metabolizes. A byproduct of this metabolism is lactic acid, which is excreted into the environment. This resultant acidic environment favors some bacterial species, while inhibiting others (Nunn and Forney 2016).

In contrast to the gut, oral cavity or vagina, urine contains very few microbes. The biomass in persons without symptoms most often ranges from 10^2 to 10^4 colony forming units per milliliter (CFU/mL) and the common threshold for a clinical UTI is $> 10^5$ CFU/mL. The microbial load is so low that even infected urine is most often crystal clear. This paucity of microbes makes urine samples, especially those obtained by suprapubic aspirate or transurethral catheter, exquisitely sensitive to contamination. This is particularly true for 16S rRNA gene sequencing, which involves amplification. Thus, extreme care must be taken during sample collection and processing to avoid environmental contamination and researchers should be aware that reagents used during processing often contain extraneous DNA, the so-called "kitome" (Stinson et al. 2019; Olomu et al. 2020).

Urobiome and lower urinary tract symptoms

Urinary tract symptoms are most commonly attributed to dysfunction in the "lower" urinary tract, typically the bladder and less commonly, the urethra. Various symptom complexes, along with specific physical findings, are used to categorize individuals into diagnostic groups. Common symptoms include urinary urgency, urinary frequency, urinary incontinence and various forms of urinary pain. Clinical algorithms for evaluation generally start with a test to detect urinary tract infection, although these clinical tests have major limitations, especially when compared to the sensitivity of modern, metagenomic and metaculturomic methods of microbial detection (Hilt et al. 2014; Price et al. 2016; Wolfe et al. 2012; Siddiqui et al. 2011; Nelson et al. 2010; Fouts et al. 2012; Khasriya et al. 2013; Coorevits et al. 2016). When urinary tract infection is suspected, most clinicians recommend anti-microbial therapy to eradicate the putative uropathogen. When urinary symptoms exist (or persist) in the absence of readily documented urinary tract infection, the symptoms are attributed to other etiologies. For example, the clinical syndrome of "overactive" bladder, defined by urgency, typically with urinary frequency and sometimes with urgency urinary incontinence, is often attributed to disorders of neuro-muscular regulation. The research that documents neuro-muscular abnormalities predates discovery of the urobiome; it is certainly plausible that microbial products affect neuro-muscular regulation of the lower urinary tract in a manner similar to GI-CNS effects (Sharon et al. 2016). This research area holds great potential for improved phenotyping and treatment for affected patients.

Unexplained urinary pain is extremely difficult for patients and their clinicians. Use of the standard urine culture has not advanced care for affected patients. Culture-independent methods are becoming available to clinicians; however, the evidence base for interpretation and treatment based on such testing is unclear. The simple presence of a microbe does not implicate that microbe in symptom causation. All current clinical efforts to eradicate urinary microbes (typically with systemic antibiotic therapy) lack precision and cause collateral urobiome effects. The suffering of affected patients should be addressed with prioritized research so as to minimize harm from antibiotic overuse.

Urobiome genomics

While early studies of the urobiome relied on 16S rRNA gene surveys of the urobiome, they could not capture the functionality present within these communities. More recently, whole genome shotgun sequencing (metagenomics) and genome sequencing of isolates have been instrumental in uncovering the functionality present in addition to providing key insights into how the bladder microbiota, for instance, differs from that of other anatomical sites. Metagenomic surveys of the urobiome include men and women with and without lower urinary tract symptoms. In comparison to other anatomical sites, relatively few metagenomic studies have focused on the urobiome (Moustafa et al. 2018; Adebayo et al. 2020; Garretto et al. 2018). Moustafa et al. conducted metagenomic sequencing of 49 individuals with UTI symptoms (Moustafa et al. 2018). In another study, metagenomic sequencing was conducted on the urobiomes of women with and without overactive bladder (OAB) symptoms (Garretto et al. 2018). Metagenomic studies can provide insight into not only the bacterial constituents of the urobiome but also the viral and eukaryotic microbial species, which cannot be captured by 16S rRNA gene surveys.

While the genomes of several urinary *Escherichia coli* isolates were available early on (Chen et al. 2006), metaculturomic methods such as the expanded quantitative urine culture (EQUC) method (Hilt et al. 2014; Price et al. 2016) have significantly enabled researchers to grow fastidious organisms from the urobiome. To date, numerous isolates of the urobiome have been cultured and their genomes sequenced. The 2018 study of Thomas-White et al. included an in-depth investigation of the genomes from 149 bladder isolates from 78 different species, representative of approximately two thirds of the bacterial diversity within the sampled bladders. These genomes were compared to 43 genomes from isolates of the vagina and gastrointestinal tract. Many of the species that inhabit the bladder are also found within the vagina but not the GI tract, and genome sequence analysis of isolates from the same individual found significant similarities, suggesting that the bladder and vaginal microbiota are interlinked (Thomas-White et al. 2018a). Besides taxonomic differences, digging deeper into the functionalities encoded by members of the bladder, vaginal, and gut microbiota also found key differences in the functionality encoded between the urogenital genomes and those from the gut (Thomas-White et al. 2018a). Taken together, these results contradict prior studies that suggested the gut serves as a reservoir for *E. coli*, and thus a source of UTIs (Yamamoto et al. 1997; Nielsen et al. 2014). Although the gut may be a source for uropathogenic infection, recent studies suggest that this may be a rare occurrence rather than the norm (Garretto et al. 2020).

Urobiome and host genetics

A recent study by Adebayo and colleagues analyzed midstream voided urine samples obtained from 1600 adult women without symptoms of UTI (Adebayo et al. 2020). These women were members of the TwinsUK cohort (Verdi et al. 2019). Because the TwinsUK cohort includes thousands of monozygotic (identical) and dizygotic (non-identical) twins, the authors were able to compare the microbiomes of these different types of twins to identify host factors that are associated with the genitourinary tract. The authors used four different analyses to test the hypothesis that genetic factors influence the genitourinary tract microbiome. First, they used heritability to quantitatively measure the contribution of multiple genes to genitourinary microbiome variability, which was both substantial and statistically significant, even after controlling for age, menopausal status, and cohabitation, as well as previous history of UTI symptoms. Some co-occurring microbial species exhibited high heritability. Second, they performed family segregation analysis and found that identical twins were less dissimilar than non-identical twins. Third, they compared ancestral origin of participants. Because the study participants were overwhelmingly of British ancestry, the authors subsampled to minimize the effect of group imbalance and found that some variance differed by ancestry. Fourth, the investigators analyzed the heritability of the core genitourinary microbiome. This analysis revealed a number of heritable species, some of which are clinically relevant. For example, infrequent uncomplicated UTIs are most often caused by *E. coli*. But, as shown by the Adebayo and co-workers, as well as another recent report (Price et al. 2019), this species is also commonly present in older asymptomatic women. Its heritability supplies evidence for the hypothesis that colonization of the genitourinary tract by *E. coli* might be a natural consequence of aging in some women and does not indicate the development of a UTI. This distinction is clinically important, as the presence of *E. coli* is often taken as evidence of UTI, especially in non-responsive women or patients with neurogenic bladders that cannot feel urinary tract symptoms. Mistaken UTI diagnoses often lead to antibiotic treatment, which can lead to resistance. It also can lead to microbiome disruption, for example, the gastrointestinal dysbiosis that permits colonization by *Clostridioides difficile*. It also appears that some heritable species can be beneficial, for example, *Lactobacillus iners*, which was recently reported to protect against post-operative UTI (Thomas-White et al. 2018b).

Closing remarks

Slow progress has plagued prevention, diagnosis and treatment of many urinary tract conditions. There is tremendous potential to improve human health through urinary microbiome research. Factors that promote or protect individuals from common lower urinary tract symptoms may one day have sound scientific rationale, rather than stale, unscientific explanations based on gender or lifestyle. As investigators join together to work in this new area of biomedical need, we will gain insights from comparisons with other human microbial communities. It is within our research to bring a scientific approach to problems such as female urinary tract infections associated with coitus, recurrent urinary tract infection, refractory bladder overactivity, recurrent renal stone disease and urothelial neoplasms. Similarities and differences will guide future hypothesis-driven research to advance patient care and improve urinary health, with the opportunity to prevent symptoms and disease, perhaps even including malignant transformation within the urinary system. Nearly all prior urinary research will benefit from re-evaluation that takes into account the presence and activities of the urobiome. As research teams assemble to advance this research, it will be critical to follow current consensus regarding research protocols and methods in order to target research resources for maximum impact.

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