Chapter

Urobiome and Bladder Cancer

Brent Gilbert and Taryn Naidoo

Abstract

Microbiome studies, fueled by the availability of high-throughput DNA-based techniques, have shown that microbiome alterations is associated with human disease including cancer. Traditionally, bladder epithelium and urine have been considered sterile in healthy individuals. This was based primarily on microbiological urine cultures, best suited for detecting aerobic, fast-growing uropathogens. Microbiome and new culturing techniques have shown that urine is not sterile but contains distinct commensal microorganisms and that alterations in commensal bladder microbes is associated with bladder cancer. This chapter focuses on identifying commensal and tumorigenic bladder bacteria, the alterations that occur in bladder cancer and impact on current treatments.

Keywords: bladder cancer, microbiome, urine cultures, schistosomiasis, urobiome

1. Introduction

The human microbiome consists of all bacteria, viral and fungal genetic material that coexists within our body [1, 2]. The microbiome is involved in a number of complex interactions with host cells, metabolic processes and the immune system which can culminate in suppression or enhancement of cancer [1]. The microbiome is not a stable entity but changes with time as people age and can be directly altered by a number of environmental and host factors [1]. To better understand the human microbiome a concerted international effort began to catalog the core microbial composition of healthy human body in the Human Microbiome Project (HMP; https://commonfund.nih.gov/hmp/) [2].

As a result of this endeavor microbial changes have been shown to be associated with multiple malignancies including colorectal, gastric, lung and breast [3]. Originally the urinary microbiota was not included in the HMP [2]. Historically it has been taught that the bladder and urine are sterile. This concept dates back to early experiments by Louis Pasteur who found that urine contained in sealed vials did not become cloudy – suggesting a lack of bacteria [4]. Over subsequent decades culturing techniques improved but only enabled detection of a limited number of bacteria, mainly aerobic, fast-growing bacteria such as *Escherichia coli* [4]. Anaerobic, slow-growing bacteria with complex nutritional needs were not detected using traditional culturing techniques. Analysis of traditional culturing techniques disregarded low bacterial yields as contaminates which further perpetuated the belief that urine was sterile and any bacteria grown must be a contaminate or an invading pathogen from genital, skin or gastrointestinal source [4].

This presumption came into refute ten years ago when it was shown that through next generation sequencing techniques (NGS) using 16 s ribosomal RNA PCR and

whole genome shotgun sequencing that urine is not sterile but replete with various microorganisms and biofilms [5]. These findings found that the healthy human bladder is colonized by a living, dynamic environment of changing microbiota. Many of the microorganisms characterized in urine were not known to cause symptomatic urinary tract infections (UTI) and are believed to be commensals. Utilization of NGS has enabled identification of new commensal and emerging uropathogens [5–8].

A drawback of NGS techniques is its inability to show the viability of bacteria identified. As a result traditional urine culturing techniques have improved to broaden the range of identifiable bacteria [9]. The use of expanded quantitative urine culture (EQUC) protocols have been incorporated into routine practice [9]. Compared to traditional culturing techniques, EQUC analyses a larger volume of urine. Samples are inoculated into multiple growth mediums and are incubated for longer periods of time under aerobic and anaerobic conditions [9]. The combined use of NGS and EQUC strategies has improved detection of urinary microbes, but distinguishing commensal from potential uropathogen continues to be defined.

The collection of microbes in the urine has been coined the urobiome and imbalances in a healthy urobiome is referred to as dysbiosis. Urinary dysbiosis is believed to contribute to a number of urological conditions including interstitial cystitis, chronic lower urinary tract symptoms and bladder cancer [10].

2. The Urobiome

2.1 Urine sampling

Urine sampling methods can dramatically impact bacterial detection and must be taken into consideration when trying to establish the normal urine microbiome. Many studies have used midstream urine samples which is not a sterile collection method. It has the potential to contain contaminants from peri-urethral or genital tract and may mislead proper characterization of the urine microbiome in favor of urogenital microbiome [8, 11]. Given anatomic differences between men and women this also poses variation of the sources of contamination. Transurethral catheters reduces the risk of contamination but it is invasive and can still potentially result in urethral bacterial contamination during catheter insertion [12]. Collecting urine via suprapubic aspiration is regarded as the most accurate method and produces less risk contamination, however one study has shown that urine microbiota obtained via transurethral catheter or suprapubic aspiration produces similar results [5]. Regardless of the specimen collection technique the urobiome has been shown to change considerably based on age, gender, race and geographic distribution [5, 8].

2.2 Defining the healthy Urobiome

Compared with vaginal and gut microbiota, the urinary microbiota has significantly less biomass. For instance, female urine is estimated to contain 10^4-10^5 colony forming units (CFU) /mL compared to 10^{12} CFU in feces [7]. There have been 562 documented species in urine and there is significant overlap with gut (64% similar) and vaginal (31% similar) microbes such that only 185 species identified are unique to urine [13, 14].

The urobiome predominantly consists of bacteria and to a lesser extent fungi, viruses and arachae. Taxonomically, microbes are classified according to phyla,

classes, orders, families, genera, and species. The phyla taxa of the urobiome is similar for men and women with the majority of bacteria belonging to the phyla Firmicutes (65% in males vs. 73% in females). The other predominate phyla include Actinobacteria (15% in males, vs. 19% in females), Bacteroidetes (10% in males vs. 3% in females) and Proteobacteria (8% in males vs. 3% in females) and 2-3% is spread across a number of low abundant phyla [11]. Urine from healthy men and women share a number of common genera with the three most prominent being Lactobacillus, Corynebacterium and Streptococcus [6, 8]. There are, however, distinct differences between the female and male urobiome.

2.3 The female Urobiome

Given the anatomical proximity between the bladder and vagina, microbial colonization of the bladder may originate or be interconnected with the vaginal microbiota. Same donor studies have revealed significant overlap of uropathogens and commensals residing in vaginal and vesicle microbiotas [15, 16]. Similar organisms included *E. coli, Streptococcus anginosus*, *Lactobacillus iners*, *Lactobacillus crispatus* and the operative taxonomic units (OTU) of *Gardnerella*, *Prevotella*, *Ureaplasma* [15, 16].

Lactobacillus is the most abundant genus found in the female urobiome and has significantly higher levels than those seen in men [7, 8, 11, 17]. Decreased levels of Lactobacillus have been associated with women of advanced age and pathological states such as UTI and Bladder cancer (BCa) [8, 18–21]. However reduced levels of *Lactobacillus* is not always a predictor of health as increased *Lactobacillus gasseri* is associated with urge urinary incontinence (UUI). *Gardnerella*, the second most abundant genera in the urobiome is also a genitourinary microbe. Gardnerella genus primarily consists of *Gardnerella vaginalis* and is a potential UTI causing uropathogen [7].

2.4 The male Urobiome

The male urobiome is less studied than the female urobiome and samples are often obtained from mid-stream urine which are prone to contamination [6]. The male microbiome is predominantly characterized by *Corynebacterium* [8] and *Streptococcus* [11] and contains less abundant *Lactobacillus* compared to women [7, 8, 11, 17]. *Pseudomonas* has also only been identified healthy men and *Staphylococcus haemolyticus* appears to have higher relative abundance in men than women [22].

2.5 Age related Urobiome changes

A number of bacteria have been shown to decrease with age. In women these include *Lactobacillus*, *Bifidobacteria*, *Sneathia*, *Shuttlewothia* and *Bacillus* [16, 19, 23]. These changes are thought to coincide with a reduction in estrogen associated with menopause. Conversely post-menopausal women show an increased relative abundance of *Mobiluncus*, *Oligella* and *Porphyromonas* [23]. Regardless of gender, individuals over 70 years have increased levels of *Jonquetella*, *Parvimonas*, *Proteiniphilum* and *Saccharofermentans* [6, 23].

2.6 The urine Virome

A number of human and bacteriophage viruses have been characterized in healthy urine specimens. Human viruses such as BK and JC polyomavirus, Herpesvirus,

Adenovirus and Anellovirus are known to reside in human urine [22, 24, 25]. These viruses have the potential to cause UTIs in immunocompromised hosts and have been associated with overactive bladders [24, 26]. Human papillomaviruses (HPVs) have also been detected in voided urine and bladder tissue [27, 28]. High risk HPV genotypes associated with cervical cancer have also been attributed to condyloma acuminatum of the bladder but there has been no direct correlation with bladder specific cancer [29, 30]. Urine may serve as a potential reservoir for local transmission of human viruses.

The vast majority of viruses in urine are bacteriophages. These viruses infect urinary bacteria such as *Lactobacillus*, *Gardnerella*, *E. coli*, *Enterococcus*, *Pseudomonas* and *Staphylococcus* [26]. Bacteriophages have been found in the urinary microbiota of both healthy women and women with UTIs [26]. Complete cataloging of bacteriophages in the urinary virome is ongoing and their contribution to urinary dysbiosis and potential association with bladder cancer is being defined [26].

2.7 The fungal and archaea Urobiome

It is difficult to ascertain if fungal and archaea cultures are naturally occurring in the urobiome or whether they are a source of contamination [31]. Midstream urine has the potential to become contaminated by nearby genitals which is known to contain fungal cultures. However, catheterized urine samples from middle aged female patients has shown to contain *Candida* spp. [7]. To date, the only archaea to be associated with urine is *Methanobrevibacter smithii* - a well-studied normal organism of the gut microbiota that it is associated with Enterobacteriaceae UTIs [32].

3. The Urobiome and bladder cancer

The relationship between the urine microbiome and cancer remains to be defined. It is possible that the urinary microbiome influences the development or progression of bladder cancer or alternatively bladder cancer influences the diversity, composition and abundance of bladder microbes.

One hypothesis is that the bladder microbiome alters the extracellular matrix which may inhibit or promote inflammation and urothelial cell carcinogenesis. When the urothelial barrier is breached, inflammatory responses promoted by opportunistic invasion of resident microbes may promote tumorigenesis. Biofilms, are microbial communities embedded in a biopolymer matrix. They are highly resistant to antibiotics and host immune responses and therefore can potentiate and propagate chronic inflammation. Bacterial biofilms have been shown to play a role in the development of a number of cancers including BCa [33]. Biofilms promote bacterial adherence, urothelial cell injury and correlates with a higher risk of developing BCa [33].

3.1 Schistosomiasis and bladder cancer

In North America and Europe approximately 90% of BCa are urothelial cell carcinoma (UCC) [34]. In Africa and the Middle East UCC bladder cancer represents 53-69% of cases and 10-40% of cases are squamous cell carcinoma (SCC) due to endemic infections of Schistosoma species [35].

Schistosoma haematobium and *Schistosoma mansoni* are common parasitic flukes that are found in fresh water primarily in sub-Sarahan Africa, South America and

sporadically in the Middle East [36]. The parasites enter the urinary tract via exposure to fresh water and lay eggs which cause inflammation and scarring of the genitourinary tract [36]. This chronic inflammation leads to squamous cell metaplasia of the urothelium and over time results in squamous cell carcinoma of the bladder [36].

The exact mechanism by which Schistosoma ova causes SCC is unclear but two factors are suspected. Firstly squamous epithelium shows greater proliferation compared to urothelial cells and hence the higher turnover of cells increase the spontaneous risk of genetic alterations that can cause cancer [37]. Secondly, chronic inflammation and exposure to environmental agents can combine to generate genotoxic urinary substances such as N-butyl-N-(4-hydroxybutyl) nitrosamine (N-Nitrosamines). N-Nitrosamines are generated in very high levels in the urine of *Schistosomiasis* patients and are known carcinogenic compounds [38]. Chronic schistosomiasis leads predominantly to SCC rather than UCC with approximately 70% of infected patients developing SCC; however many patients will have both SCC and UCC [39]. Interestingly, alterations in the urobiome may influence *Schistosomiasis* related bladder cancer. Individuals infected with Schistosomiasis and had urine colonized with *Fusobacterium, Sphingobacterium* or *Enterococcus* were more likely to progress to bladder cancer [40]. It is suggested that strains of bacteria which mediate the formation of N-nitrosamines contribute to schistosomiasis-induced bladder cancer [40].

3.2 Urothelial cancer and the Urobiome

The urobiome and its role in bladder cancer is an emerging field of investigation and the interpretation of findings is often difficult to appreciate given the various host, environmental and sampling factors that contribute and can significantly alter the composition of the urobiome. There is also a great deal of variation when it comes to specimen processing, sequencing targets, taxonomy assignment databases and statistical analysis performed. These issues must be taken into consideration when interpreting findings. Most of our understanding so far regarding the urobiome in bladder cancer is generated from retrospective cohort and case control studies. There have been very few prospective or higher level research studies to date [41].

Bacterial diversity within a sample is quantified by several statistical methods and is expressed as alpha-diversity (α-diversity). Whereas beta-diversity (β-diversity) is a measure of diversity between two environments ie; bladder cancer vs. no cancer. So far there is no consensus regarding BCa urine/tissue having greater or less bacterial diversity or species richness [41]. However there are certain genus/species which have been reported to be more common in BCa specimens (**Table 1**).

3.3 Microbial changes in urothelial cancer

A number of studies have identified higher abundances of **Acinetobacter** genus in tissue and urine of bladder cancer patients [20, 21, 42, 43]. **Acinetobacter** is a complex genus consisting of gram-negative, anaerobic, biofilm forming species [44]. **Acinetobacter** is capable of adhering, degrading and invading urothelial barriers. This allows it to evade antibiotics and host immune responses and possibly promote carcinogenesis directly through urothelial injury or alterations of cell-cycle proliferation, or indirectly enabling invasion of other opportunistic tumorigenic uropathogens [44, 45]. **Actinomyces** genus is a common urogenital commensal that is often seen in women and has the potential to cause suppurative and granulomatous opportunistic infections. *Actinomyces*, in particular *A. europaeus,* is increased in the urine of BCa

Genera	Sample	Bladder Cancer Trend	Known functional effect
Acinetobacter	Urine Tissue	↑ ↑	Biofilm forming genus Invasive pathogen that can degrade phospholipid membranes Associated with urothelial cancer in other species
Actinomyces (*A. europaeus)*	Urine	↑	Opportunistic uropathogen
Actinotignum	Urine	↑	Opportunistic uropathogen elevated in women
Anaerococcus	Urine	↑	Biofilm producer Opportunistic uropathogen Extracellular matrix remodeling
Aeromonas	Urine	↑	Secrete extracellular proteases
Tepidomonas	Urine	↑	Secrete extracellular proteases
Pseudomonas	Urine	↑	Secrete extracellular proteases Secretes anti-tumor exotoxin-A immunotoxin Elevated in BCG Responders
Burkholderia	Urine Tissue	↑ ↑	Inhibits tumorigenesis by blocking CTLA-4 signaling
Sphingomonas	Urine Tissue	↑	Degrades aromatic compounds
Escherichia-Shigella	Urine Tissue	↓ ↑	Uropathogen Secretes genotoxic colibactin toxin Elevated in BCG responders
Klebsiella	Tissue	↑	Uropathogen Secretes genotoxic colibactin toxin Elevated in BCG responders
Lactobacillus	Urine Tissue	↓ ↓	Probiotic with Anti-tumor properties Secretes lactic acid and H2O2 Competitively excludes uropathogens Increases effectiveness of epirubicin
Bifidobacterium	Urine	↓	Induces apoptosis via multiple pathways
Roseomonas	Urine	↓	Improves epithelial barriers Suppresses *S. Aureus* Immunomodulation through lipid mediated TNFa-receptor signaling
Corynebacterium	Urine	↑ and ↓	Opportunistic uropathogen Hydrolyzes lipids yielding anti-bacterial free fatty acids.
Veillonella	Urine IDC Urine	↓ ↑	Utilizes lactic acid produced by Lactobacillus Reduces nitrate levels by converting it to nitrite
Streptococcus	Urine	↑ and ↓	Large genus with multiple species Species have tumorigenic and anti-tumorigenic potentials

IDC – indwelling catheter; BCG – Bacille Calmette-Guerin; TNFα – Tumor Necrosis Factor α; CTLA-4 -Cytotoxic T-lymphocyte associated protein 4.

Table 1.
Summary of genera that have been identified in more than one study and implicated in attenuation or progression of bladder cancer.

patients [18]. **Actinotignum**, an Actinomyces-like organism is a urine commensal and opportunistic uropathogen that was only elevated in female BCa patients [46]. *Anaerococcus*, *Tepidomonas and Pseudomonas* are elevated in voided BCa urine they and can induce inflammation and remodeling of the extracellular matrix (ECM) which provides access to the suburothelial space for opportunistic tumorigenic uropathogens and may contribute to BCa onset, progression and relapse [42, 47, 48]. *Aeromonas*, *Tepidomonas* and *Pseudomonas* secrete extracellular proteases which disrupts the ECM [42, 48]. **Pseudomonas** may also have anti-tumor potential via the production of an exotoxin-A immunotoxin which shows specific and efficacious anti-tumor cytoxocity [49]. To further support anti-tumor properties of *Pseudomonas* it is elevated in the urine of Bacille Calmette-Guerin (BCG) responders compared to BCG non-responders [47]. *Burkholderia* is a urinary commensal that is increased in BCa tissue and urine [47, 50]. Its role in BCa is unknown but it may inhibit tumorigenesis by blocking CTLA-4 signaling [51]. *Sphingomonas* is elevated in BCa tissue and urine and known to degrade carcinogenic aromatic compounds [20, 21, 42, 43, 52].

Corynebacterium is an abundant urinary commensal that is elevated in male urine and reported to be elevated in BCa urine by most studies [43, 50, 53, 54]. When detected in BCa urine it has been associated with high grade NMBC and MBC [8, 53, 54]. *Corynebacterium* species hydrolyze lipids and release free fatty acids with anti-bacterial activity and are also potential opportunistic uropathogens [55]. The role of *Corynebacterium* in the urobiome and possible contribution to BCa is not known. It is unclear if *Eschericihia-Shigella* and *Klebsiella* genus are elevated in BCa [20, 21, 50, 56, 57]. *Eschericihia-Shigella* and *Klebsiella* are uropathogens capable of influencing tumorigenesis by producing genotoxic colibactin; a toxin that induces DNA strand breaks resulting in genomic instability [58]. *Eschericihia-Shigella* is also elevated in BCG responder urine which may indicate that certain bacteria may be needed to be present to prime the immune response for optimal BCG effect [56].

The majority of bacteria that are decreased in BCa tend to beneficial. *Lactobacillus* is a large genus that contributes significantly to the urine commensal population [8, 11]. *Lactobacillus* is urogenital commensal that is found in greater numbers in women compared to men and reduces post-menopause [23]. These microbes play an important role in regulating UTIs through the production of lactic acid and hydrogen peroxide, colonizing resistance and competitively excluding pathogens [59]. The protective role of *Lactobacillus* may translate into reduced tumorigenesis which may help to explain the sex-disparity of bladder cancer [60]. *Bifidobacterium* [18] has been shown to induce apoptosis through intrinsic and extrinsic pathways that involves increasing expression of Fas, FasL, Cyt-C, Caspase-3, Caspase-9 and lowering cancer proliferation proteins such as PCNA, PFK-B, HKK-1, PKM2 [61]. *Roseomonas* is an immunomodulation genus that can improve epithelial barriers and suppresses competing bacteria such as *Staphylococcus aureus* through lipid mediated TNFα- receptor signaling [62].

Veillonella and *Streptococcus* genus are both urinary commensals that have protective potentials which have been shown to be decreased and increased in BCa [18, 43, 47, 54, 56, 57]. *Veillonella* is a probiotic that is capable of using lactic acid produced by Lactobacillus and converting nitrates to nitrites [63]. Higher nitrate levels are thought to contribute to N-nitrosamines formation and increased risk of bladder cancer [64] *Streptococcus* is a large genus which contains a number of species with tumorigenic and anti-tumorigenic potentials [65, 66]. Further investigation at the species level is needed to clarify the role that Streptococcus plays in BCa.

4. The Urobiome and bladder cancer treatment

4.1 Probiotics

Before the microbiome era researchers were aware that oral administration of probiotic bacteria could potentially reduce incidence and recurrence of bladder cancer [67–69]. Specifically, *Lactobacillus casei* and *Lactobacillus rhamnosus* were shown to have cytotoxic effect on BCa cells and inhibited BCa growth. The mechanism may be propagated through NK cell activity but this is not known for sure [70]. A randomized controlled trial compared standard intravesical epirubicin alone with epirubicin plus one year oral intake of *L. casei* strain Shirota in patients who had undergone resection of intermediate-risk NMIBC. A statistically significant 15% absolute reduction in long-term tumor recurrence was seen in the group that received the oral probiotic. However the dropout rate of the probiotic group was 3.5 times the control group and this called into question the reliability of this study [71]. Given that there is renewed interest in microbial influence in BCa and we have new tools to evaluate the microbiome these studies may need to be re-investigated.

4.2 Bacille Calmette-Guerin (BCG) treatment

Intravesical BCG instillations have been a mainstay of adjuvant therapy for high and intermediate risk of non-muscle invasive bladder cancer (NMIBC). BCG failure leading to disease recurrence and progression remains a significant clinical issue. Recent microbiome research has shown that *Escherichia-Shigella*, *Pseudomonas*, and *Serratia* were significantly more abundant in the urine of BCG responsive patients compared to non-responders [56]. The belief is that uropathogenic bacteria is needed to help prime the immune directed response of BCG [72].

The complete mechanism by which BCG controls BCa proliferation still remains unclear. However, BCG is believed to bind fibronectin sites on the urothelial wall and become internalized through RAS and PI3K-PTEN dependent micropinocytosis process. The tumor-specific immune response increases in intensity over the course of the treatment period. A number of urinary microbes can bind fibronectin and have the potential to out-compete BCG for fibronectin binding and attenuate BCG efficacy. One such microbe is *L. iners* which is predominantly found in females and is shown to preferably bind fibronectin and decrease BCG efficacy [73]. Screening urine microbiome and targeting fibronectin binding microbes ahead of BCG treatment may lead to improved treatment outcomes.

4.3 The Urobiome and immunotherapy

The role of the microbiome may extend to advanced BCa disease management. Immunotherapy agents, particularly those utilizing the PD-1/PD-L1 axis have seen increased use in advanced BCa. The efficacy of these agents have been associated with composition of the gut microbiome. It is plausible that the composition of the urobiome may also influence the response of anti-PD1/PDL1 therapy. It has been reported that antibiotic use within one month of starting atezolizumab is associated with reduced overall survival in locally advanced and metastatic platinum-refractory BCa treated by atezolizumab [74]. An additional study has also shown that antibiotic use during pembrolizumab neoadjuvant immunotherapy in MIBC was associated with greater relapse and poorer outcomes [75].

5. Conclusion

The dogma that urine is sterile is no longer acceptable as recent technological advances have shown that urine contains a number of commensal microbes. However, there is still much to learn about the urobiome regarding its composition and function during homeostasis and disease. A number of cross-sectional and case–control studies have identified changes in the urobiome associated with bladder cancer. However, further research using appropriate confounding controls and employing multi-omic approaches is required to clarify the implications of these taxonomic differences and their role in bladder cancer with the hope of establishing diagnostic or prognostic microbial markers and improved therapeutic modalities and outcomes.

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Author details

Brent Gilbert[1,2*] and Taryn Naidoo[2]

1 The University of Newcastle School of Medicine, Newcastle NSW, Australia

2 Surgical Sciences Department, Hunter New England Health, John Hunter Hospital, Newcastle, NSW, Australia

*Address all correspondence to: brentgilbert@gmail.com; brent.gilbert@health.nsw.gov.au

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