Characterization of the Female Urinary Microbiota and Their Association with the Female Bladder Uroepithelium

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CHARACTERIZATION OF
THE FEMALE URINARY MICROBIOTA
AND THEIR ASSOCIATION WITH
THE FEMALE BLADDER UROEPITHELIUM

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THE FACULTY OF THE GRADUATE SCHOOL
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BY
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<th>Abbreviation</th>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>DMEM/F-12</td>
<td>Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>EQUC</td>
<td>Expanded Quantitative Urine Culture</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>HPLC-MS</td>
<td>High Pressure Liquid Chromatography Mass Spectroscopy</td>
</tr>
<tr>
<td>GBS</td>
<td>Group B <em>Streptococcus</em></td>
</tr>
<tr>
<td>IRB</td>
<td>Institutional Review Board</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile Range</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectroscopy</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>MV</td>
<td>Midstream Voided</td>
</tr>
<tr>
<td>OAB</td>
<td>Overactive Bladder Syndrome</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase-Chain Reaction</td>
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<tr>
<td>PFDI</td>
<td>Pelvic Floor Distress Inventory</td>
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LIST OF ABBREVIATIONS (CONTINUED)

SPA  Supra-Pubic Aspirate
TUC  Transurethral Catheter
UPEC Uropathogenic *Escherichia coli*
UTI  Urinary Tract Infection
ABSTRACT

The current clinical dogma assumes that urine is sterile in the absence of clinically relevant infection. However, recent evidence has demonstrated the existence of a female urinary microbiota in women with and without lower urinary tract symptoms. With the knowledge that the lower urinary tract possesses its own unique microbiota, I hypothesize that certain bacterial species of the female microbiota may be the cause or play a role in lower urinary tract syndromes, such as overactive bladder syndrome (OAB). About 40-50% of OAB patients do not respond to conventional anti-muscarinic and beta-3 adrenergic agonist drug treatment. One possible explanation for this lack of treatment response is a dysbiosis of urinary microbiota. To determine if women with OAB have a dysbiotic urinary microbiota, our group developed an expanded quantitative urine culture (EQUC) protocol to culture transurethral catheter urine specimens obtained from women with and without OAB. EQUC revealed differences in the female urinary microbiota in women with and without OAB. Given evidence of planktonic bacteria in the urine, I hypothesized that bacteria also may be associated with the urothelium. To test this hypothesis, I examined urothelial cells shed into urine for the presence of adherent and/or intracellular bacteria. I used a new protocol that was able to determine which bacteria associate with shed urothelial cells, but could not distinguish between adherent or intracellular bacteria. By identifying differences in the urinary microbiota between women with different disease/health states, and by determining which of those bacteria
associate with the urothelium, one can begin to understand how the female urinary microbiota could contribute to lower urinary tract disorders.
CHAPTER ONE
INTRODUCTION

Urinary health disorders affect many people. For example, overactive bladder syndrome (OAB) affects about 15% of adult women with increasing incidence with age (Hartmann et al., 2009). The exact etiology of OAB is not known, but one cause is thought to be a neuro-muscular dysfunction; thus, the treatment options for OAB are the administration of anti-muscarinic drugs or beta-3 adrenergic receptor agonists to relieve the symptoms (Michel & Chapple, 2009). However, the side effects (such as severe dry mouth or urinary retention) can be uncomfortable for the patient and only 50-60% of patients have their symptoms resolved (Santos & Telo, 2010, Chapple et al., 2013). Since anti-muscarinic and beta-3 adrenergic agonist treatments aimed at relaxing the bladder are ineffective in a large percentage of OAB sufferers, it is likely that there are etiologies outside of neuro-muscular dysfunction (Nitti et al., 2010).

Emerging DNA sequencing and culture evidence indicates that microbial communities exist within the bladder (Siddiqui et al., 2011, Fouts et al., 2012, Nelson et al., 2012, Wolfe et al., 2012, Khasriya et al., 2013, Lewis et al., 2013, Brubaker et al., 2014, Hilt et al., 2014, Pearce et al., 2014). With this evidence that bacterial DNA (microbiome) and live bacteria (microbiota) exist within the bladders of women with OAB and of women without OAB, it is possible that microbes represent a new etiology
or a contributing factor for OAB. For example, women who do not respond to OAB therapies might not suffer from OAB; instead, a causative or contributing bacterium might not be a known uropathogen or it might be present at lower colony forming units (CFU)/mL than the threshold of $10^5$ CFU/mL used in standard practice since the 1950s (Kass, 1956, Kass, 1957). Alternatively, women who do not respond may have a dysbiosis of their microbiota. How bacteria cause OAB symptoms is not known, but bacteria present in the bladder could interact with the urothelium such that the detrusor urinae muscle is induced to contract and initiate, exacerbate or propagate OAB symptoms.

The overall hypothesis of this present study was that the female urinary microbiota are different in women with and without OAB. The aims to address the overall hypothesis of the present study were to: (1) isolate and identify the bacteria that comprise the female urinary microbiota and (2) determine if the bacterial species of the female urinary microbiota are associated with the human urothelium.
Female Lower Urinary Tract Biology

The lower urinary tract consists of the following anatomical sites: the ureters, the bladder, and the urethra. In a female, the ureters connect the renal pelvis to the bladder, which is then connected to the vulva by the urethra (Martini FH, 2009).

The ureters are muscular tubes that extend inferiorly from the renal pelvis and penetrate the posterior wall of the bladder where urine is deposited. The bladder is a hollow muscular organ that functions as a temporary storage unit for urine. When the bladder is empty, it is decompressed; as the bladder fills with urine, it takes on a spherical shape. The area of the bladder where the urethra attaches is known as the trigone. This trigone acts as a funnel that channels urine through the urethra as the bladder contracts. The urethra, which is about 3-5 cm in females, connects the neck of the bladder to the vulva or exterior (Martini FH, 2009).

Each anatomical site has its own characteristic histology. The ureters consist of three layers: an inner mucosa lining also known as urothelium, a muscular layer made up of smooth muscle, and an outer connective tissue layer (Martini FH, 2009, Birder & Andersson, 2013). The bladder also contains three layers. First, there is the mucosa layer, comprised of the urothelium and the lamina propria (Martini FH, 2009, Birder & Andersson, 2013). Next is the muscularis propria, which contains the detrusor urinae muscle; when contracted, it compresses the bladder to expel the urine into the urethra (Martini FH, 2009, Birder & Andersson, 2013). Finally there is the adventitia/serosa layer composed of connective tissue (Martini FH, 2009, Birder & Andersson, 2013). At the
junction of the bladder and urethra, there is a circular band of skeletal muscle known as the urethral sphincter that remains in a relaxed muscle state until it is voluntarily contracted to allow urination. The urethra is lined with a transitional epithelium and is surrounded by layers of smooth muscle (Martini FH, 2009).

The detrusor urinae muscle can be stimulated by a variety of receptors by both the parasympathetic and sympathetic nervous systems (Martini FH, 2009). Two types of receptors are stimulated via the parasympathetic nervous system: the cholinergic nicotinic and muscarinic receptors; both are stimulated by the neurotransmitter acetylcholine (ACh), which induces an activation or contraction effect on the detrusor urinae muscle (Martini FH, 2009). The type of receptor stimulated via the sympathetic nervous system is the adrenergic beta-receptor. The adrenergic beta-receptor is stimulated by the neurotransmitters epinephrine and norepinephrine and has an inhibitory or relaxation effect on the detrusor urinae muscle (Martini FH, 2009, Goldenberg, 2012).

Recent evidence shows urothelial cells that comprise the urothelium can be stimulated via the same receptors found on the detrusor urinae muscle. These receptors include the adrenergic beta-receptors and the cholinergic nicotinic and muscarinic receptors. Thus, urothelial cells can both be targets of these receptors’ neurotransmitters and release various mediators to influence detrusor urinae muscle cell contraction (Birder & Andersson, 2013).
History of Clinical Microbiology Urine Culture

The current clinical dogma assumes that urine is sterile in the absence of clinically relevant infection. Since the 1950’s, the clinical practice for detecting infection in the bladder, including urinary tract infection, cystitis and pyelonephritis, has been based on a method that detects bacterial species present in mid-stream urine at greater than or equal to \(10^5\) colony forming units (CFU)/mL (Kass, 1956, Kass, 1957). However, this threshold of \(\geq 10^5\) CFU/mL was not designed to detect infection in the bladder. Instead, it was originally set as a threshold to detect kidney infection or pyelonephritis (Kass, 1956, Kass, 1957), which is characterized by chills, fever, flank pain and dysuria (Beeson, 1955, Kass, 1956). In the 1950’s, Dr. Edward Kass, an infectious disease physician, sought a method to prevent post-operative sepsis in patients undergoing kidney surgery. To achieve his goal, he needed a reliable test that could detect uropathogens in urine collected via a non-invasive procedure. He chose midstream urine and identified the threshold of \(\geq 10^5\) CFU/mL as adequate for the task (Kass, 1956, Kass, 1957).

Yet, many studies have provided evidence that this threshold is insufficient to detect significant infection of the lower urinary tract in all types of patient populations (Stamm et al., 1982, Stark & Maki, 1984, Lipsky et al., 1987, Maskell, 2010, Hooton et al., 2013, Khasriya et al., 2013). Dr. William Stamm (1982) demonstrated that \(10^5\) of a known uropathogen in the midstream urine of women was indicative of lower urinary tract infection (Stamm et al., 1982), while Dr. Benjamin Lipsky (1987) showed that a threshold of \(10^3\) CFU/mL of a known uropathogen in midstream urine was indicative of lower urinary tract infection in men (Lipsky et al., 1987). Similarly, Dr. Randall Stark
(1984) determined that a bacterium present at lower than $10^5$ CFU/mL was indicative of lower urinary tract infection in catheterized patients (Stark & Maki, 1984).

At about the same time, Dr. Rosalind Maskell (1981-1988) performed scientifically rigorous studies that provided compelling evidence to disprove the dogma that urine was sterile in the absence of a clinically relevant infection (Maskell, 2010). In one of her studies, Dr. Maskell collected supra-pubic aspirate (SPA) urine specimens from women suffering from a whole host of lower urinary tract disorders, including dysuria and interstitial cystitis (Maskell, 2010). She then plated the SPA samples and incubated the plates in different conditions for a longer period. She explained:

“Overnight incubation in air of cultures on a primary isolation medium does not detect organisms other than the aerobic pathogens. Many organisms with other requirements, for example anaerobes and CO$_2$-requiring bacteria and those species that need longer incubation, are well recognised as pathogens in sites other than the urinary tract (Maskell, 2010)”

Dr. Maskell showed the presence of bacteria in these SPA samples and hypothesized that other bacterial species are likely present in the urinary tract. She further hypothesized that it is either a dysbiosis of commensal flora and/or an unknown uropathogen that is causing these women to suffer from lower urinary tract disorders other than a urinary tract infection (UTI) (Maskell, 2010).

Dr. Maskell was not the only one to hypothesize that bacteria may influence symptoms in lower urinary tract disorders. Dr. Thomas Hooton and colleagues (2013)
obtained transurethral catheter (TUC) specimens from women suffering from cystitis and found evidence of many Gram-positive bacteria, such as lactobacilli, staphylococci, streptococci and *Gardnerella vaginalis* (Hooton *et al.*, 2013). Hooton and colleagues did not go into detail about whether these Gram-positive bacteria are a consequence or a cause of cystitis, but they did suggest a re-evaluation of the use of midstream urine cultures to make an accurate diagnosis of a patient with lower urinary tract symptoms and to consider the idea the bladder possesses a resident bacteria flora (Hooton *et al.*, 2013).

**Overactive Bladder Syndrome**

Urinary health disorders affect many people. For example, overactive bladder syndrome (OAB) affects about 15% of adult women with increasing incidence with age (Hartmann *et al.*, 2009). OAB is characterized by symptoms of urinary urgency, often with frequency and urgency incontinence, nocturia and a negative standard urine culture (Haylen *et al.*, 2010).

The exact etiology of OAB is not known, but one cause is thought to be a neurological disorder due to increased release of the neurotransmitter ACh, which binds to cholinergic nicotinic and cholinergic muscarinic receptors on the detrusor urinae muscle and causes contractions and the feeling of urgency (Martini FH, 2009, Michel & Chapple, 2009).

One treatment for OAB is the administration of anti-muscarinic drugs (e.g., Solifenacin) to prevent the binding of ACh to the cholinergic muscarinic receptor and therefore relieve the symptoms (Michel & Chapple, 2009, Nitti *et al.*, 2010). However,
uncomfortable side effects (such as severe dry mouth) are causes for reduced compliance. Furthermore, only 50-60% of patients have their symptoms resolved (responders), while 40-50% of patients retain symptoms (non-responders) (Santos & Telo, 2010).

Another more recent treatment option for OAB is the administration of a beta-3 adrenergic receptor agonist known as Mirabegron. This agonist binds to beta-3 adrenergic receptors and causes the detrusor muscle to relax (Goldenberg, 2012, Afeli et al., 2013). This relaxation of the detrusor muscle counteracts the overstimulation of cholinergic muscarinic receptors. However, a major side effect with Mirabegron is urinary retention due to too much relaxation of the detrusor muscle (Goldenberg, 2012). Moreover, the response rate to Mirabegron is similar to that of Solifenacin (Chapple et al., 2013, Abrams et al., 2014).

Since both anti-muscarinic and beta-3 adrenergic agonist treatments aimed at relaxing the bladder are ineffective in a large percentage of OAB sufferers, it is likely that there are etiologies and/or causes outside of neuro-muscular dysfunction (Nitti et al., 2010).

The Female Urinary Microbiome/Microbiota

Recent DNA sequencing and culture evidence reveals the presence of microbial communities within the bladder (Siddiqui et al., 2011, Fouts et al., 2012, Nelson et al., 2012, Wolfe et al., 2012, Khasriya et al., 2013, Lewis et al., 2013, Brubaker et al., 2014,
Hilt et al., 2014, Pearce et al., 2014). These data demonstrate the existence of the urinary microbiome (bacterial DNA) and the urinary microbiota (live bacteria).

Various investigators have used culture-independent 16S rRNA gene sequencing to acquire evidence of diverse bacteria that are not routinely cultured by clinical microbiology laboratories in the mid-stream urine of women and men (Nelson et al., 2010, Dong et al., 2011, Siddiqui et al., 2011, Nelson et al., 2012).

Wolfe and co-workers (2012) went a step further, providing definitive evidence of bacterial DNA in the bladders of women. They used 16S rRNA gene sequencing to identify bacterial DNA present in ‘culture-negative’ urine specimens collected from women diagnosed with pelvic organ prolapse and/or urinary incontinence and from women without urinary symptoms. In this study, urine was collected via three methods: clean, catch midstream voided (MV), transurethral catheter (TUC) and suprapubic aspirate (SPA). The bacterial DNA communities detected in paired TUC and SPA samples were similar, while the MV samples contained a mixture of urinary and genital tract bacterial communities. Because the SPA samples were obtained directly from the bladder, these researchers concluded that urine in the bladder contained bacterial DNA. Since the SPA and TUC samples were similar, they concluded that TUC samples were representative of bacterial communities present in the bladder (Wolfe et al., 2012).

**Intracellular Bacteria and Urothelium**

Many bacterial species reported to cause UTI colonize the urinary tract. The only way to successfully colonize is to associate with the urothelium. A key step in
pathogenesis for major uropathogens, such as *Proteus mirabilis, Klebsiella pneumoniae*, and *Escherichia coli*, is their association with the urothelium (Coker et al., 2000, Struve et al., 2008, Hunstad & Justice, 2010). In the case of *Klebsiella pneumoniae* and *Escherichia coli*, they both express type 1 fimbriae that attach to urothelial cell receptors; this allows them to be taken up intracellularly (Struve et al., 2008, Hunstad & Justice, 2010). *Proteus mirabilis* expresses many types of fimbriae, which permits it to attach to diverse urothelial cell receptors (Pearson et al., 2008). Other bacterial species shown to associate with the urothelium and reported to cause a UTI include *Streptococcus agalactiae* or Group B *Streptococcus* (GBS) and *Enterococcus faecalis* (Ulett et al., 2010, Horsley et al., 2013).

Bacterial species that associate with the urothelium are of interest because they are likely to play a role in lower urinary tract disorders such as OAB. With evidence of a urinary microbiota (Hilt et al., 2014) and evidence that certain bacterial species associate with shed uroepithelial cells in the urine (Horsley et al., 2013, Khasriya et al., 2013), it is reasonable to hypothesize that some of these bacteria associate with the urothelium and that these associations may play a role in lower urinary tract disorders.
CHAPTER TWO

METHODS AND MATERIALS

Patients and Sample Collection: Following Loyola institutional review board (IRB) approval for all phases of this project, participants gave verbal and written consent for the collection and analysis of their urine for research purposes. Participants were women undergoing OAB treatment and a comparison group of women undergoing benign gynecologic surgery (controls). Participants' symptoms were characterized with the Pelvic Floor Distress Inventory (PFDI), a self-completed, validated symptom questionnaire (Barber et al., 2006). All participants were without clinical evidence of urinary tract infection (i.e., standard urine culture negative and absence of clinical UTI diagnosis). Urine was collected via transurethral catheter from participants for the period March 2013 to April 2014 at the Female Pelvic Medicine and Reconstructive Surgery center of Loyola University Medical Center. A portion of each urine sample was placed in a BD Vacutainer Plus C&S Preservative Tube (Becton Dickinson and Co; Franklin Lakes, NJ) and sent to the clinical microbiology lab for quantitative culture. A separate portion of the urine sample, to be used for sequencing, was placed at 4°C for no more than 4 h following collection. To this portion, 10% AssayAssure (Thermo Scientific; Waltham, MA) was added before freezing at −80°C.
**Standard Urine Culture:** The clinical microbiology laboratory staff processed each catheterized urine sample using the standard culture procedure. Standard urine culture was performed by inoculating 0.001 ml of urine onto a 5% sheep blood agar plate (BAP) and MacConkey agars (BD BBL™ Prepared Plated Media, Becton Dickinson and 94 Co; Sparks, MD) and streaking the entire plate surface to obtain quantitative colony counts. The plates were incubated aerobically at 35°C for 24 h (Figure 1). Each separate morphological colony type was counted and identified in any amount. The detection level was $10^3$ CFU/ml, represented by 1 colony of growth on either plate. If no growth was observed, the culture was reported as “no growth” (of bacteria at lowest dilution, i.e., 1:1000).

**Expanded Quantitative Urine Culture (EQUC):** Each catheterized urine sample also was processed using the EQUC procedure (Hilt et al., 2014). For EQUC, 0.1 ml of urine was inoculated onto BAP, chocolate and colistin, and nalidixic acid (CNA) agars (BD BBL™ Prepared Plated Media), streaked for quantitation, and incubated in 5% CO$_2$ at 35°C for 48 h. A second set of BAPs, were each inoculated with 0.1 ml of urine and incubated in room atmosphere at 35°C and 30°C for 48 h. Next, 0.1 ml of urine was inoculated onto each of two CDC anaerobe 5% sheep blood agar plates (BD BBL™ Prepared Plated Media) and incubated in either a Campy gas mixture (5% O$_2$, 10% CO$_2$, 85% N) or under anaerobic conditions at 35°C for 48 h. The detection level was 10 CFU/ml, represented by 1 colony of growth on any of the plates. Finally, to detect any bacterial species that may be present at quantities lower than 10 CFU/ml, 1.0 ml of urine was placed in thioglycolate medium (BD BBL™ Prepared Tubed Media) and incubated aerobically at
Figure 1-Outline of Standard Urine and Expanded Quantitative Urine Culture
35°C for 5 days. If growth was visually detected in the thioglycolate medium, the medium was mixed and a few drops were plated on BAP and CDC anaerobe 5% sheep blood agar plates for isolation and incubated aerobically and anaerobically at 35°C for 48 h (Figure 1). Each morphologically distinct colony type was isolated on a different plate of the same media to prepare a pure culture that was used for identification. Because no unique bacterial species were detected in three of the EQUC conditions (Campy Gas mixture-5% O2, 10% CO2, 85% N at 37°C for 48 hours, Aerobic at 30°C for 5 days, and Thioglycolate medium Aerobic for 5 days), I removed those conditions from the EQUC protocol as of December 2013

**Identification of Pure Isolates:** Matrix assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was used to identify each morphologically distinct colony. The direct colony method was performed. Using toothpicks, we applied a small portion of a single isolated colony to the surface of a 96-spot, polished, stainless steel target plate (Bruker Daltonik GmbH, Leipzig, Germany) in a manner that created a thin bacterial film. The spot was left to dry at room temperature for 1 min., whereupon 1.0 µl of 70% formic acid was applied to each sample and allowed to dry at room temperature for 10 min. Then, 1.0 µl of the matrix solution, comprised of saturated α-cyano-4-hydrocinnamic acid (Bruker Daltonik) in an organic solvent (High-Pressure Liquid Chromatography-Mass Spectrometry [HPLC-MS]-grade water, 100% Trifluoroacetic Acid, and Acetonitrile; Fluka) was then applied to each sample and allowed to cocrystallize at room temperature for 10 min. The prepared sample target was placed in the MicroFlex LT mass spectrometer (Bruker Daltonik), and the results were analyzed by
MALDI Biotyper 3.0 software (Bruker Daltonik). A bacterial quality control strain (Escherichia coli DH5α) was included in each analysis. A single measurement was performed once for each culture isolate.

**MALDI Data Analyses:** MALDI Biotyper 3.0 software Realtime Classification was used to analyze the samples. In the Realtime Classification program, log score identification criteria are used as follows. A score between 2.000 and 3.000 is species-level identification, a score between 1.700 and 1.999 is genus-level identification, and a score that is below 1.700 is an unreliable identification. A Realtime Classification log score was given for each bacterial isolate sample for every condition from which it was isolated.

**DNA Isolation, PCR Amplification and 16S rRNA Amplicon Sequencing:** Genomic DNA was extracted from urine using previously validated protocols (Wolfe et al., 2012, Yuan et al., 2012). Briefly, 1 ml of urine was centrifuged at 13,500 rpm for 10 min and the resulting pellet was resuspended in 200 µl of filter-sterilized buffer consisting of 20 mM Tris-Cl (pH 8), 2 mM Ethylenediaminetetraacetic Acid (EDTA), 1.2% Triton X-100, and 20 µg/ml lysozyme and supplemented with 30 µl of filter-sterilized mutanolysin (5,000 U/ml, Sigma-Aldrich; St. Louis, MO). The mixture was incubated for 1 h at 37°C and the lysates were processed through the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The DNA was eluted into 50 µl of AE buffer (pH 8.0) and stored at −20°C. The variable region 4 (V4) of the bacterial 16S rRNA gene in each DNA sample was amplified and sequenced using a custom
protocol developed for the MiSeq desktop sequencer (Illumina; San Diego, CA). Briefly, the 16S rRNA V4 region was amplified in a two-step nested polymerase-chain reaction (PCR) protocol using the universal 515F and 806R primers, which were modified to contain the Illumina adapter sequences. Amplicons were analyzed by gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen). Extraction- and PCR-negative controls were included in all steps to assess potential DNA contamination. DNA samples were diluted to 10 nM, pooled, and sequenced using the MiSeq personal sequencer platform using a paired-end 2× 251-bp reagent cartridge. Raw sequences were processed using the open-source program mothur, v1.31.2 (Kozich et al., 2013). Paired ends were joined and contigs of incorrect length (<285 bp or >300 bp) and/or contigs that contained ambiguous bases were removed. Sequences were aligned using the SILVA database, and chimeric sequences were removed with UCHIME (Edgar et al., 2011). Sequences were classified using a naive Bayesian classifier and the RDP 16S rRNA gene training set (v9). Sequences that could not be classified to the bacterial genus level were removed from analysis.

**Statistical Analyses:** Statistical analyses of the microbiota data were performed using SAS software version 9.3. The frequency of detected genera and species was compared between groups (OAB v Control), using either Pearson chi-square or Fisher’s exact tests, depending on assumption validity. The Wilcoxon rank sum tests were used to compare the median abundance for the cultured genera between groups. No adjustments for multiple comparisons were made since these analyses were considered descriptive.
**Filtered Urine Culture:** Each urine specimen (10 mL) was placed through a filtration system (Figure 2). The first 5 mL of urine was filtered through a Nucleopore filter with a pore size 5.0 µm (Whatman). To ensure that free bacteria pass through the filter, a 5.0mL phosphate buffered saline (PBS) wash was performed. The 5.0 µm filter, which captured the urothelial cells (20 -100 µm) (Croft *et al.*, 1979, Bostwick & Cheng, 2008), was placed on a blood plate and incubated in a 5% CO₂ incubator at 37°C for 48 hours. The filtrate, containing planktonic bacteria, was subjected to the EQUC protocol (above in Materials and Methods). All colonies were counted and any morphologically distinct colony was isolated onto a fresh plate composed of the same medium to prepare a pure culture to be used for identification with MALDI-TOF MS. The second 5 mL of urine was filtered through a Nucleopore filter with a pore size of 0.2 µm, as described above. All bacteria and urothelial cells were captured on the filter (Hobbie *et al.*, 1977, Bernhardt *et al.*, 1991). The filter and filtrate from the 0.2 µm arm were handled as described above.
Figure 2 - Outline of Filtered Urine Culture Protocol
**Antibiotic Protection Assay:** As a control, the EQUC protocol was performed on the urine samples (as described in Aim 1) to identify the bacterial species present without treatment. The urine sample (50 mL) was centrifuged at 400 x g at room temperature for 10 minutes. The supernatant was removed and the pellet was washed in 1.0 mL of PBS. The 1.0 mL of solution was split into two 0.5mL solutions and centrifuged at the same speed and time. Then, each pellet was re-suspended in either 1.0 mL of PBS or 1.0 mL of an antibiotic cocktail (200 ug/mL Gentamicin, 100 units/mL Penicillin, 100 mg/mL Streptomycin) in PBS and then let sit for 2 hours to kill any planktonic or adherent bacteria. The 1.0 mL solution for each condition was split into two 0.5 mL solutions and centrifuged at the same speed and time. Then, the pellets either were re-suspended in 1.0 mL of PBS or 1.0 mL of 0.1% Triton-X-100 in PBS and let sit for 5 minutes to lyse the cells. This procedure resulted in a total of four conditions (PBS-PBS, PBS-Triton X, Antibiotics-PBS, Antibiotics-Triton X); all four were subjected to the EQUC protocol (Figure 3). All colonies were counted and any morphologically distinct colony was isolated onto a fresh plate composed of the same medium to prepare a pure culture for identification with MALDI-TOF MS.
Figure 3- Outline of Antibiotic Protection Assay
**Human Urothelial Cell/UPEC Infection Assay:** An infection assay with an immortalized human urothelial cell line infected with uropathogenic *Escherichia coli* (UPEC) was performed and then subjected to the antibiotic protection assay as a proof of principle (**Figure 4**). The infection assay with the immortalized human urothelial cell line was designed and optimized by Dr. Meghan Pearce. Dr. Phong Lee established the immortalized human urothelial cell line from a female non-malignant bladder. Four days prior to infection of urothelial cells, strains of UPEC were streaked onto Lysogeny Broth (LB) agar and incubated overnight at 37°C. Then a single colony of UPEC strain of interest was inoculated into 5 mL of liquid LB and incubated overnight at 37°C shaking. Next, a subculture of 1:1000 of the UPEC into fresh liquid LB was done and allowed to incubate at 37°C overnight with no shaking. The day before infection, this subculture process was repeated. To prepare the human urothelial cells, the day before infection, cells were counted with a hemocytometer and then diluted to 1x10^5 cells/mL in tissue culture media. In triplicate, 1 mL aliquots of urothelial cells were placed into a 24-well tissue culture treated plate and incubated overnight at 37°C in 5% CO_2 to allow the cells to adhere to the well. The day of infection, the human urothelial cells were examined to see that they had adhered to the wells. The old media was aspirated out and the cells were washed 3 times with 1 mL PBS. Then 1.0 mL of Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12)/5% Fetal Bovine Serum (FBS) was added to each well and allowed to incubate for 30min. To obtain a rough estimate of the multiplicity of infection (MOI) for UPEC, one well of urothelial cells was trypsinized and the number of cells
Figure 4- Outline of Human Urothelial Cell/UPEC Infection Assay
counted. The UPEC culture was either diluted or concentration to so that there would be 10 UPEC cells for every 1 urothelial cell. Once this UPEC MOI of 10 was prepared, 1.0 mL of this inoculum was added to each of three wells containing urothelial cells. As a negative control, one well of cells was not inoculated with UPEC. The 24-well plate with cells was centrifuged at 1400 rpm for 5 min to initiate contact of the UPEC with the urothelial cells. The plate was incubated at 37°C in 5% CO₂ for 2 hours. Once the 2-hour infection was completed, the cells in each well were trypsinized (1 uninfected well and 3 infected wells) and run separately through the Antibiotic Protection Assay. As a control, to determine the average intracellular count per well, the infection assay was continued. First, the human urothelial cells and UPEC were treated for 2 hours with the same antibiotic cocktail used in the antibiotic protection assay (200 ug/mL Gentamicin, 100 units/mL Penicillin, 100 mg/mL Streptomycin). Second, the cells were washed 3 times with 1.0 mL of PBS and then treated with 1.0 mL of 0.1% Triton-X-100 in PBS and let sit for 5 minutes to lyse the cells. Finally, each well was plated with serial dilutions onto LB agar plates to determine the average intracellular count per well.

**Tissue-Culture Adherence:** The following protocol was designed and optimized by Dr. Meghan Pearce (Figure 5). Each urine sample (20 mL) was centrifuged at 400 x g for 10 minutes. The resultant pellet was then re-suspended in tissue culture media and split into microtiter plate wells with or without antibiotics (200 ug/mL Gentamicin, 100 units/mL Penicillin, 100 mg/mL Streptomycin). The plate was then placed in a 5% CO₂-enriched incubator at 37°C for overnight incubation. Observations of human cells, cell debris, and
1. Observations: Human cells and cell debris (amount and cell morphology varies by urine), bacteria
2. Gentle PBS wash
3. Add 0.5ml 0.1% tx-100
4. Spin to “pellet” (13500rpm, 10min)
5. Plate on blood agar 37deg, 5% CO2 48hr

Figure 5- Outline of Tissue Culture Adherence Assay
bacteria in the wells were observed the next day. Then, the wells were subjected to a gentle PBS wash to identify human urothelial cells that remain adherent to the plastic. Following the wash, 0.5 mL of 0.1% Triton-X-100 was added to the cells for five minutes to lyse the urothelial cells and the mixture centrifuged at 13,500 rpm for 10 minutes. This resultant pellet was re-suspended in PBS and plated on a blood agar plate. This blood agar plate was then be placed in a 5% CO\textsubscript{2} incubator at 37°C for 48 hours. All colonies were counted and any morphologically distinct colony was isolated onto a fresh plate composed of the same medium to prepare a pure culture to be used for identification with MALDI-TOF MS.

**Antibiotic Sensitivities of Bacterial Isolates:** For the bacterial isolates that appeared to be intracellular, a control experiment for antibiotic sensitivity was performed. The bacterial isolates were tested for sensitivity to the antibiotics used in the cocktail (200 \text{ug/mL} Gentamicin, 100 \text{units/mL} Penicillin, 100 \text{mg/mL} Streptomycin).

**Culture of Bladder Biopsy:** Following Loyola institutional review board (IRB) approval for all phases of this project, participants gave verbal and written consent for the collection and analysis of their urine and tissue for research purposes. The following samples were collected from participants: transurethral catheter (TUC) urine, suprapubic aspirate (SPA) urine and two small tissue biopsies of the bladder urothelium. EQUC was performed on the TUC and SPA urine samples (as described in Aim 1) to identify the bacterial species present. The tissue biopsies were washed twice with 1.0 mL of saline.
After the second wash, each tissue biopsy was ground with a sterile mortar and pestle in 1.0 mL of saline. Then 100 µL of the saline mixture was subjected to the EQUC protocol.
CHAPTER THREE

ISOLATION AND IDENTIFICATION OF BACTERIA THAT COMPRIZE THE FEMALE URINARY MICROBIOTA

INTRODUCTION

16S rRNA gene sequence analysis of urine specimens has revealed the existence of bacterial DNA in urine obtained from women diagnosed with pelvic organ prolapse and/or urinary incontinence, as well as from women without urinary symptoms or prolapse. Thus, a female urinary microbiome exists (Wolfe et al., 2012).

In addition, others and we have shown that these bacterial sequences represent live bacteria; therefore, female urinary microbiota exist (Khasriya et al., 2013, Hilt et al., 2014). These urinary microbiota were detected using expanded versions of the standard urine culture. Specifically our group developed and performed an expanded quantitative urine culture (EQUC) on TUC urines to culture bacterial genera detected by 16S rRNA gene sequencing in previous studies. The bacterial genera being cultured matched the previous sequencing data demonstrating the presence of a urinary microbiota (Hilt et al., 2014).

With the existence of female urinary microbiota, one needs to determine if they play a role in lower urinary tract disorders such as OAB. One diagnostic criterion for OAB is a negative test for UTI - typically a standard urine culture. This culture is when one inoculates 0.001 mL of urine to blood and MacConkey agar plates and then incubates
aerobically at 35°C for 24 hours. This standard technique is geared toward detecting known uropathogens at the $10^5$ CFU/mL threshold, consistent with the 1950’s intent of Dr. Kass. Since the previous 16S rRNA sequencing and recent culture data of urine both demonstrate the presence of bacteria that may not be known uropathogens and/or are present at levels lower than the threshold, an expanded urine culture technique is needed to fully assess the differences in the female urinary microbiota of women with and without OAB.
RESULTS

**Standard Urine Culture vs. Expanded Quantitative Urine Culture (EQUC)**

A total of 189 transurethral catheter urine specimens were collected from 61 women with OAB and 52 women without OAB. All urine specimens were processed through standard and expanded quantitative urine culture (EQUC) techniques.

Most (153/189) urine specimens grew bacterial species with the EQUC approach. Yet, 139 of the 153 urine specimens that grew bacterial species were deemed ‘No Growth’ (**Table 1**) of bacteria at the lowest dilution, i.e., 1:1000) by the clinical microbiology lab. These data demonstrate that bacterial species are present in ‘culture-negative’ urines and the standard urine culture has a false-negative rate of ~90%. One specimen in particular demonstrates the value of the expanded quantitative culture (**Figure 6**). To date, a total of 608 bacterial isolates that make up 116 diverse bacterial species have been isolated from these urine specimens (**Table 1**). The median number of different bacterial species per urine specimen was three (**Table 1**).
Table 1- Summary of Urine Specimens

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Total Specimens</td>
<td>189</td>
</tr>
<tr>
<td>Percentage of Bacterial Growth</td>
<td>80.95% (153/189)</td>
</tr>
<tr>
<td>Percentage Deemed No Growth by Standard Culture Technique</td>
<td>90.85% (139/153)</td>
</tr>
<tr>
<td>Number of Bacterial Isolates</td>
<td>708</td>
</tr>
<tr>
<td>Diverse Number of Species</td>
<td>116</td>
</tr>
<tr>
<td>Median Number of Species Per Urine</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 6- 4-Week OAB Patient Urine. Urine specimen subjected to: (A) Standard Culture Blood plate incubated 72 hours at 35°C. This routine plate was finalized as ‘No Growth’ (of bacteria at the lowest dilution of 1:1000) at 24 hours. (B) Expanded Culture Blood plate incubated 72 hours at 35°C.
Female Urinary Microbiota in Baseline Urine Specimens in Women with and without OAB.

A subset of 104 baseline urine specimens were subjected to the standard and expanded quantitative urine cultures, 52 from women without OAB (Control) and 52 from women with OAB. 33 of the 52 (63.46%) control baseline specimens grew bacterial species, while 45 of the 52 (86.54%) OAB baseline specimens grew bacterial species.

Overall the OAB baseline urine specimens had a greater number of bacterial isolates than the control baseline urine specimens. Only 82 bacterial isolates were isolated from the control specimens, whereas 261 bacterial isolates were isolated from the OAB specimens (p= <0.0001). The median number of different bacterial species isolated per control urine specimen was one [Interquartile Range (IQR)=0-2]), while the median number of different bacterial species isolated per OAB urine specimen was four (IQR=1-7).

My results show that the OAB female urinary microbiota is richer than the control female urinary microbiota. To date, 35 unique bacterial species make up the microbiota of the controls, while 80 unique bacterial species comprise the microbiota of the symptomatic OAB patients (Figure 7).
Figure 7- Rarefaction Analysis of Unique Bacterial Species. Each unique bacterial species isolated is documented in each patient cohort as patients are recruited. (OAB N=52, Control N=52). 35 unique bacterial species comprise the control female urinary microbiota, while 80 unique bacterial species comprise the OAB female urinary microbiota.
Statistical Analyses of Baseline Urine Cultures of Women with and without OAB.

The Pearson chi-square or Fisher’s exact tests were performed on the subset of 104 baseline urine specimens to determine differences in frequency of the genera and species isolated in each cohort.

Seven bacterial genera (*Actinobaculum, Actinomyces, Aerococcus, Corynebacterium, Gardnerella, Oligella* and *Streptococcus*) were isolated more frequently in the OAB cohort compared to the control cohort (*Figure 8, Table 2*). Three of these bacterial genera were solely isolated from the OAB cohort (*Actinobaculum, Aerococcus*, and *Oligella*); concentrations ranged from 10 CFU/mL to 100,000 CFU/mL.

The following bacterial species were isolated more frequently in OAB urine specimens than from control urine specimens: *Actinobaculum schaalii, Aerococcus urinae, Corynebacterium coyleae, Corynebacterium riegelii, Gardnerella vaginalis, Lactobacillus gasseri, Oligella urethralis*, and *Streptococcus anginosus* (*Figure 9, Table 2*). The bacterial species *Lactobacillus crispatus* was isolated more frequently in the control urine specimens than the OAB urine specimens (*Figure 9, Table 2*).
Figure 8 - Frequency of Bacterial Genera in Each Cohort. Comparison of the percentage a given bacterial genus was isolated in either an OAB urine specimen (black bars, N=52 urine specimens) or a control urine specimen (white bars, N=52 urine specimens). Pearson’s Chi-square and Fisher’s Exact Test were performed to determine statistically significant differences in isolated bacterial genera (* >0.05, **>0.001).
**Figure 9- Frequency of Bacterial Species in Each Cohort.** Percentage a given bacterial species was isolated in either an OAB urine specimen (black bars, N=52 urine specimens) or a control urine specimen (white bars, N=52 urine specimens). Pearson’s Chi-square and Fisher’s Exact Test were performed to determine statistically significantly differences in isolated bacterial species (* >0.05, **>0.001).
Table 2- P-values of Significant Bacterial Genera and Species

<table>
<thead>
<tr>
<th>GENERA</th>
<th>OAB</th>
<th>Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobaculum</td>
<td>8 (15.4%)</td>
<td>0 (0%)</td>
<td>0.0058</td>
</tr>
<tr>
<td>Actinomyces*</td>
<td>16 (30.8%)</td>
<td>2 (3.9%)</td>
<td>4.50E-04</td>
</tr>
<tr>
<td>Aerococcus*</td>
<td>12 (23.1%)</td>
<td>0 (0%)</td>
<td>2.39E-04</td>
</tr>
<tr>
<td>Corynebacterium*</td>
<td>13 (25%)</td>
<td>3 (5.8%)</td>
<td>0.0125</td>
</tr>
<tr>
<td>Gardnereilla*</td>
<td>13 (25%)</td>
<td>3 (5.8%)</td>
<td>0.0125</td>
</tr>
<tr>
<td>Oligella</td>
<td>6 (11.5%)</td>
<td>0 (0%)</td>
<td>0.0268</td>
</tr>
<tr>
<td>Streptococcus*</td>
<td>23 (44.2%)</td>
<td>10 (19.2%)</td>
<td>0.0109</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>OAB</th>
<th>Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. schwalii</td>
<td>7 (13.5%)</td>
<td>0 (0%)</td>
<td>0.0126</td>
</tr>
<tr>
<td>A. urinace*</td>
<td>11 (21.2%)</td>
<td>0 (0%)</td>
<td>5.42E-04</td>
</tr>
<tr>
<td>C. coagulate</td>
<td>8 (15.4%)</td>
<td>0 (0%)</td>
<td>0.0058</td>
</tr>
<tr>
<td>C. riegelii</td>
<td>6 (11.5%)</td>
<td>0 (0%)</td>
<td>0.0268</td>
</tr>
<tr>
<td>G. vaginalis*</td>
<td>11 (21.2%)</td>
<td>2 (3.9%)</td>
<td>0.015</td>
</tr>
<tr>
<td>L. crispatus*</td>
<td>2 (3.8%)</td>
<td>8 (15.4%)</td>
<td>0.0923</td>
</tr>
<tr>
<td>L. gasser*</td>
<td>13 (25%)</td>
<td>5 (9.6%)</td>
<td>0.0676</td>
</tr>
<tr>
<td>S. anginosus*</td>
<td>18 (34.6%)</td>
<td>4 (7.7%)</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

The whole number value represents the number of urine specimens in each cohort from which a given bacterial genus/species was cultured. The (%) is the percentage of the total urine specimens in each cohort from which a given bacterial genus/species was cultured. The p-value is the significant value for the Fischer’s Exact Test. The * designates significance reached for the Pearson Chi-Square Test.
Comparison of Urine Culture and 16S rRNA Sequencing Data.

A subset of the baseline urine specimens subjected to EQUC (29% 30/105) was also examined by 16S rRNA sequencing. In each of these 30 urine specimens, bacterial DNA was detected; therefore, they were sequenced. Dr. Meghan Pearce performed all the sequencing preparation and analyses.

Extensive overlap was observed in terms of the bacterial genera detected with both sequencing and EQUC (Figure 10). A total of 18 different genera were cultured with EQUC; all but Trueperella were detected by sequencing. These data provide additional evidence that the sequenced genera of the urinary microbiome represent live bacteria that make up the urinary microbiota.

In some urine specimens, EQUC detected genera not detected by sequencing (Figure 10). Yet, these genera were sequenced in other urine specimens inferring that they can be amplified with the universal primers used for sequencing. In these particular urines, the genera detected by EQUC were in CFU concentrations lower than that which could be detected by sequencing ($10^1$-$10^3$).

Five genera (Atopobium, Prevotella, Rhodanobacter, Sneathia, and Veillonella) were only detected by sequencing (Figure 10). These data suggest that EQUC is limited in its ability to detect these organisms.
Figure 10- Comparison of Bacterial Genera Detected by EQUC and 16S rRNA

**Sequencing.** A comparison of the bacterial genera detected by sequencing and culture of urine specimens (N=30). Each square was color-coded based on whether the bacterial genera were detected by sequence only (green), EQUC only (red), sequence and EQUC (yellow) or neither sequence nor EQUC (grey).
Recent evidence demonstrates the existence of urinary microbiota (Hilt et al., 2014, Pearce et al., 2014). Thus, live bacteria are present in the bladder, but it is not clear if the bacteria associate with the bladder urothelium. Bacterial species that associate with the urothelium are of interest because they are more likely to play a role in OAB symptoms.

Previous studies have shown many uropathogens can attach to and invade urothelial cells (Coker et al., 2000, Struve et al., 2008, Hunstad & Justice, 2010); therefore, it is reasonable to hypothesize that some other urinary bacteria do likewise. This hypothesis is supported by evidence that bacterial species obtained from standard culture negative patients with lower urinary tract symptoms are closely associated to urothelial cells (Horsley et al., 2013, Khasriya et al., 2013).

There are two methods one could use in order to identify bacterial species of the urinary microbiota that associate with the urothelium. The first is to culture bladder biopsy tissue. The second method to is to examine bacterial communities associating with shed urothelial cells in urine (Horsley et al., 2013, Khasriya et al., 2013).
In the latter experiment, a protocol was developed to optimize the second method for future study use and to avoid the invasive procedure of a bladder biopsy to distinguish between the bacterial populations (planktonic, adherent, intracellular) of the urinary microbiota. The protocol combined three different assays to get an accurate picture of the different bacterial populations in the urinary microbiota. These three assays or arms of the protocol are the filtered urine culture (Figure 2), an antibiotic protection assay (Figure 3) and a tissue-culture adherence assay (Figure 5).
RESULTS

Bacterial Species of the Female Urinary Microbiota and Association with Bladder Biopsy Tissue.

Four sets of bladder biopsies and urines (TUC and SPA) were obtained from patients undergoing surgery at the Female Pelvic Medicine and Reconstructive Surgery center of Loyola University Medical Center. All four patient’s urine and biopsy samples were cultured using EQUC.

Three of the four patients did not have any growth in all the conditions (Table 3). In contrast, bacteria were cultured from the urines and biopsies of one patient (010) (Table 3). The TUC and SPA urine specimens contained significant and matching counts of *Klebsiella pneumoniae* and *Staphylococcus aureus*. Both organisms were isolated from the bladder biopsies, but in lower counts. The bladder biopsies of Patient 010 also grew additional organisms (*Lactobacillus gasseri*, *Enterococcus faecalis*, and *Enterococcus faecium*) that were not found in the TUC and SPA samples. The failure to detect these organisms in the TUC and SPA samples may be due to low CFUs that cannot be detected with EQUC, and/or the fact that both *K. pneumoniae* and *S. aureus* overgrew the plates.
### Table 3- Bladder Biopsy Tissue Culture Results

<table>
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<tbody>
<tr>
<td><strong>Culture Condition</strong></td>
<td><strong>Organism</strong></td>
</tr>
<tr>
<td>TUC</td>
<td>None</td>
</tr>
<tr>
<td>SPA</td>
<td>None</td>
</tr>
<tr>
<td>Biopsy 1</td>
<td>None</td>
</tr>
<tr>
<td>Biopsy 2</td>
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<tbody>
<tr>
<td><strong>Culture Condition</strong></td>
<td><strong>Organism</strong></td>
</tr>
<tr>
<td>TUC</td>
<td>None</td>
</tr>
<tr>
<td>Biopsy 1</td>
<td>None</td>
</tr>
<tr>
<td>Biopsy 2</td>
<td>None</td>
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</table>

<table>
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</thead>
<tbody>
<tr>
<td><strong>Culture Condition</strong></td>
<td><strong>Organism</strong></td>
</tr>
<tr>
<td>TUC</td>
<td>None</td>
</tr>
<tr>
<td>Biopsy 1</td>
<td>None</td>
</tr>
<tr>
<td>Biopsy 2</td>
<td>None</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Patient-010</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Culture Condition</strong></td>
<td><strong>Organism</strong></td>
</tr>
</tbody>
</table>
| TUC | *Klebsiella pneumoniae*  
*Staphylococcus aureus* | $>10^5$  
$>10^5$ |
| SPA | *Klebsiella pneumoniae*  
*Staphylococcus aureus* | $>10^5$  
$>10^5$ |
| Biopsy 1 | *Klebsiella pneumoniae*  
*Staphylococcus aureus*  
*Lactobacillus gasseri*  
*Enterococcus faecium*  
*Enterococcus faecalis* | $10^2$  
$10^2$  
$10^1$  
$10^1$  
$10^1$ |
| Biopsy 2 | *Klebsiella pneumoniae*  
*Staphylococcus aureus*  
*Lactobacillus gasseri*  
*Enterococcus faecalis* | $10^2$  
$10^2$  
$10^1$  
$10^1$ |
Antibiotic Protection Assay’s Ability to Distinguish Between Populations of Female Urinary Microbiota and their Associations with Urothelial Cells.

To distinguish between bacteria that associate with shed urothelial cells and planktonic bacteria, we adapted a previously published antibiotic protection assay (Khasriya et al., 2013) (Figure 3). This assay also should distinguish between bacteria associated with the urothelial cells into subpopulations of bacteria that adhere to the cell surface (PBS arms) and the bacteria that are intracellular (lysed arms) (Figure 3).

A total of 10 practice urine specimens collected via transurethral catheter were used to optimize this Antibiotic Protection Assay arm (Table 4). These practice urines were leftover urine specimens destined to be discarded after clinical evaluation of patients at the Female Pelvic Medicine and Reconstructive Surgery center of Loyola University Medical Center.

Four of the 10 practice urines (#7, 8, 12, 16) had ‘No Growth’ in any of the conditions. However, bacteria were cultured from the other six practice urines and the assay was able to distinguish between the bacterial species that were planktonic and those that associated with shed urothelial cells. For example in both practice urines 10 and 11, $10^6$ CFU of *Escherichia coli* was detected in the EQUC control. Once the urine was placed through the assay and concentrated, there were 5 and 10 CFU/mL of *Escherichia coli*, respectively, suggesting that a large amount of the *Escherichia coli* in these urine specimens was planktonic while a small subset associated with the urothelial cells.

Practice Urine 9 (Table 4) had ‘No Growth’ in the control EQUC arm, but *Escherichia coli* was detected in low CFU’s once the urine specimen was concentrated
(PBS-treated). Thus, bacteria can be present in lower CFU counts than the thresholds for the standard urine culture approach and even the EQUC protocol.

Practice Urine 15 (Table 4) had the same amount of *Klebsiella pneumoniae* and *Enterococcus faecalis* in all three arms of the experiment. Since both organisms were cultured in the antibiotic-treated arm, antibiotic sensitivities were performed on both organisms. The *Klebsiella pneumoniae* and *Enterococcus faecalis* were both resistant to the antibiotic cocktail (200 ug/mL Gentamicin, 100 units/mL Penicillin, 100 mg/mL Streptomycin). This resistance was most likely acquired naturally within the patient or was present in the bacteria before entering the patient. The resistance exhibited by these organisms demonstrates the limit of this assay. In this urine, we are unable to distinguish between bacteria that are planktonic and bacteria that are associated with the urothelial cell.
### Table 4- Antibiotic Protection Assay Practice Urine Results

<table>
<thead>
<tr>
<th>Practice Urine</th>
<th>EQUC</th>
<th>PBS-PBS</th>
<th>PBS-Triton X</th>
<th>Antibiotic-PBS</th>
<th>Antibiotic-Triton X</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>26 E. coli</td>
<td>20 E. coli</td>
<td>2 E. coli</td>
<td>2 E. coli</td>
</tr>
<tr>
<td>10</td>
<td>10^9 E. coli</td>
<td>5 E. coli</td>
<td>5 E. coli</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>10^9 E. coli</td>
<td>10 E. coli</td>
<td>10 E. coli</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>4x1 K. pneumoniae 30 S. pneumoniae 10 C. ureae</td>
<td>25 K. pneumoniae 30 S. pneumoniae 10 C. ureae</td>
<td>35 K. pneumoniae 20 S. pneumoniae 5 C. ureae</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>10 C. ureae</td>
<td>100 K. pneumoniae</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>10^3 K. pneumoniae 10^3 S. pneumoniae 10^3 N. gonorrhoeae</td>
<td>10^3 K. pneumoniae 10^3 S. pneumoniae 10^3 N. gonorrhoeae</td>
<td>10^3 K. pneumoniae 10^3 S. pneumoniae 10^3 N. gonorrhoeae</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Culture results for the antibiotic protection assay for practice urines 7-16. The – designates a negative or No Growth result of the urine in that arm of the assay. The number in front of the organism represents the number of colony forming units (CFU)/mL of urine for which that organism was present.
**Antibiotic Protection Assay In Detection of Intracellular Bacteria**

In the six of 10 practice urines that grew bacterial species, I was able to distinguish between the bacterial species that were planktonic and those that were associated with shed urothelial cells. However, from these data, I was unable to distinguish between bacteria that were attached to urothelial cells and those that were intracellular. But, a proof of principle assay had not been performed to determine whether this protocol could detect intracellular bacteria. Therefore, I grew immortalized urothelial cells and infected them with UPEC, known to be taken up intracellularly by these cells (Hunstad & Justice, 2010) and then ran the cells with UPEC through the Antibiotic Protection Assay Arm. If the assay were detecting intracellular bacteria in urothelial cells, then I would detect UPEC in the antibiotic treatment branch of the assay (Figure 3).

In both instances (Run 1 and Run 2), the Antibiotic Protection Assay Arm did not detect intracellular UPEC in the immortalized human urothelial cells (Table 5). However, the Human Urothelial Cell/UPEC Infection Assay arm for Run 1 and Run 2 found an average intracellular count of 330 CFU/mL and 220 CFU/mL of UPEC per well, respectively. These data suggest that the current Antibiotic Protection Assay is unable to detect intracellular bacteria.
Table 5- Human Urothelial Cell/UPEC Infection Assay Results

<table>
<thead>
<tr>
<th>Run 1</th>
<th>Treatment Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^5 cells/well MOI=10</td>
<td>Before Assay</td>
</tr>
<tr>
<td>Uninfected Cells</td>
<td>NG</td>
</tr>
<tr>
<td>Well 1-NU14</td>
<td>10^5</td>
</tr>
<tr>
<td>Well 2-NU14</td>
<td>10^5</td>
</tr>
<tr>
<td>Well 3-NU14</td>
<td>10^5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Run 2</th>
<th>Treatment Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^5 Cells/well MOI=10</td>
<td>Before Assay</td>
</tr>
<tr>
<td>Uninfected Cells</td>
<td>NG</td>
</tr>
<tr>
<td>Well 1-NU14</td>
<td>10^5</td>
</tr>
<tr>
<td>Well 2-NU14</td>
<td>10^5</td>
</tr>
<tr>
<td>Well 3-NU14</td>
<td>10^5</td>
</tr>
</tbody>
</table>

Summary of the two runs of the proof of principle Human Urothelial Cell/UPEC Infections Assay. The NG designates a negative or No Growth result for that condition. The number designates the amount of cells per well cultured in that condition.
Adherent vs. Intracellular Bacterial Populations

In order to further differentiate between bacterial populations that either adhere to the urothelial cell surface or are taken up intracellularly, a total of four urine specimens were placed through both the antibiotic protection and tissue culture adherence assay arms of the protocol. The antibiotic protection assay is able to distinguish between bacteria that are planktonic and bacteria that are associated with the urothelial cells (Figure 3). The tissue-culture adherence assay arm is designed to further characterize the bacterial populations that associate with the cells into bacteria that adhere to the cell surface and the bacteria that are taken up intracellularly (Figure 5).

In the antibiotic protection assay arm, bacteria were detected in all four urine specimens in the no antibiotic treatment arm (Table 6). All bacterial species detected in the four urines were also detected in the EQUC control; however, they were detected in two-three logs lower CFU counts. These data suggest that the bacterial species in the no antibiotic treatment arm are associated with the shed urothelial cells and are not planktonic. In the tissue-culture adherence assay, two of the four urine specimens in the no antibiotic arm were not plated for culture (Table 6). This was due to the fact that the pH indicator in the tissue culture media had changed, indicating a large amount of cell debris being present in the wells.

In all four specimens, there was no detection of intracellular bacteria in the antibiotic protection assay. However, with the tissue-culture adherence assay, bacteria were cultured from the antibiotic condition in two of the four urine specimens, suggesting
that the bacteria may have been intracellular (Table 6). To confirm that these organisms were intracellular, antibiotic sensitivities should have been performed.
### Table 6-Antibiotic Protection and Tissue-Culture Adherence Results

<table>
<thead>
<tr>
<th>Practice Urine</th>
<th>Antibiotic Protection Assay</th>
<th>Tissue-Culture Adherence Assay</th>
<th>EQUC Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibiotic -</td>
<td>Antibiotic +</td>
<td>Antibiotic -</td>
</tr>
</tbody>
</table>
| 17            | *Aerococcus urinae*  
*Lactobacillus jensenii*  
*Streptococcus agalactiae*  
*Actinomyces naesl* | None | N/A | None | *Aerococcus urinae*  
*Lactobacillus jensenii*  
*Streptococcus agalactiae*  
*Actinomyces naesl* |
| 18            | *Streptococcus anginosus* | None | *Streptococcus anginosus* | *Micrococcus luteus* | *Streptococcus anginosus* |
| 19            | *Staphylococcus epidermidis*  
*Rohila dentocariens* | None | N/A | *Actinomyces odontolyticus*  
*Actinomyces oris*  
*Neisseria elongata*  
*Acinetobacter flavescens*  
*Rohila dentocariens*  
*Rohila mucilaginosa*  
*Streptococcus anginosus*  
*Streptococcus cristaus*  
*Streptococcus salivarius* | *Actinomyces oris*  
*Rohila dentocariens*  
*Streptococcus anginosus*  
*Streptococcus cristaus* |
| 20            | *Lactobacillus johnsonii*  
*Staphylococcus capsulatus*  
*Lactobacillus crispatus* | None | *Brevibacterium Casei* | None | *Staphylococcus capsulatus*  
*Lactobacillus crispatus* |

Summary of culture results for practice urines 17-20 using both the antibiotic protection and tissue-culture adherence assays. The None represents no bacteria were grown in that culture condition in the assay. The N/A designates the culture was not performed due to overgrowth of bacteria in the tissue-culture adherence assay. Every practice urine had an EQUC control culture performed.
CHAPTER FOUR

DISCUSSION

The data presented here support the existence of the female urinary microbiota and further demonstrate differences in the female urinary microbiota in individuals with and without OAB.

One diagnostic criterion for women who suffer from OAB is a negative standard urine culture. The implication of the negative standard culture is that the individual does not suffer from a clinically relevant urinary tract infection and that the symptoms are not caused by a bacterium (Haylen et al., 2010). However, recent work done by others and our group support the hypothesis that standard urine culture is not effective in detecting most of the bacterial taxa present in urine (Khasriya et al., 2013, Hilt et al., 2014). Khasriya and colleagues (2013) compared standard cultures with cultures of centrifuged urinary sediment from MV samples from men and women. They were able to culture a large number of undetected bacteria in the urinary sediment compared to the standard culture (Khasriya et al., 2013). Our group demonstrated in our recent publication and here with this work that the standard urine culture had a false-negative rate of ~90% (Table 1). It is clear that the standard urine culture with a threshold of $10^5$ CFU and incubation in aerobic conditions for 24 hours is not sufficient for complete and accurate diagnosis, because it cannot culture a large portion of urinary bacteria. All the evidence suggests that many culture-negative standard urine cultures are not actually culture-
negative and demonstrates the need to reevaluate the standard urine culture technique 
and to expand it to help women suffering from lower urinary tract disorders other than 
UTI.

Our group is not the first to use an expanded culture condition to suggest the re-
evaluation of the standard urine culture technique. As mentioned in the literature review, Dr. Rosalind Maskell plated SPA samples on various agar plates and incubated the plates 
in a variety of conditions for a longer period of time, a protocol similar to our EQUC protocol. She was able to culture organisms including *Gardnerella vaginalis*, *Lactobacillus* spp., and fastidious *Streptococci* (Maskell, 2010). Although specific 
species are not mentioned in her text, we found *Gardnerella* a lot of *Lactobacillus* and 
*Streptococcus* species in the female urinary microbiota.

The female urinary microbiota in women with OAB are different from the 
microbiota of women without OAB. Several bacterial genera were more frequently 
cultured from the urine of women with OAB, including *Actinobaculum, Actinomyces, 
Aerococcus*, and *Oligella*. Interestingly, many of these genera contain emerging 
uropathogens, including *Actinobaculum schaali, Aerococcus urinae*, and *Oligella 
urethralis* (Bank et al., 2010, Zimmermann et al., 2012, Dabkowski et al., 2013, 
Rasmussen, 2013), which also were seen more frequently in the urinary microbiota of 
women with OAB. Whether these bacteria contribute to OAB is unknown at this time and 
requires further research.

Another interesting bacterial species that was more frequently cultured in the 
OAB cohort than the non-OAB cohort was *Gardnerella vaginalis* (Figure 9). This
bacterium is a facultative anaerobe isolated from the vaginal tract and known to be a biomarker of the vaginal dysbiosis called bacterial vaginosis (Liu et al., 2013). At this time, it is not known if the *G. vaginalis* isolates from the OAB cohort are pathogenic, but it has been shown previously that *G. vaginalis* can be cultured from the vaginal tracts of women with and without bacterial vaginosis (Hyman *et al.*, 2005, Harwich *et al.*, 2010). More research must be performed to determine if *G. vaginalis* isolates from both the OAB and non-OAB cohorts are either pathogenic or symbiotic strains of *G. vaginalis*.

Intriguingly, the genus *Lactobacillus* was cultured frequently in both the OAB and non-OAB cohorts. The bacteria in this genus are lactic acid-producing, facultative anaerobic bacteria known to play protective roles in the vaginal tract by decreasing pH and producing various bacteriostatic/cidal compounds (Redondo-Lopez *et al.*, 1990, Kaewsrichan *et al.*, 2006). However, the *Lactobacillus* species isolated more frequently from either cohort was different. *L. gasseri* was cultured more frequently from the OAB cohort and *L. crispatus* was cultured more frequently from the non-OAB cohort (Figure 9). At this time, more research must be performed to determine if this difference is a consequence of OAB or whether it contributes to OAB symptoms. Stapleton and colleagues (2011) showed that *L. crispatus* could be used as a probiotic for recurrent UTI suggesting that it could play protective role in the female urinary microbiota (Stapleton *et al.*, 2011).

Finally, these data demonstrate that live culture results match that of sequencing results (Figure 10). 16S rRNA gene sequencing gives broad view of the urinary bacterial communities, while culturing live bacteria provides a more focused view.
The evidence for urinary microbiota is compelling, but whether these bacteria associate with the bladder urothelium remains uncertain. Bacterial species that associate with the urothelium are of particular interest because they are more likely to play a role in OAB symptoms. The bacteria present in the urinary microbiota could associate with the bladder urothelium in two ways: either (1) by adherence to the surface of the urothelial cells or (2) by invasion of the urothelial cell. The possibility also exists that some urinary bacteria are planktonic and do not associate with the urothelium. Such bacteria could influence the urothelium via secreted products.

There are two methods one could use to identify bacterial species of the urinary microbiota that associate with the urothelium. The first is to go straight to the source and culture bladder biopsy tissue; however, this method is invasive and it is difficult to obtain samples on a regular basis. The second method is to examine bacterial communities associated with shed urothelial cells in urine (Horsley et al., 2013, Khasriya et al., 2013).

The first method, culture of bladder tissue biopsy, provided some evidence that the urinary microbiota (or some bacteria in the urinary microbiota) associate with the human urothelium. I obtained and cultured four sets of bladder biopsy tissue with urine samples. I was unable to culture any bacteria in the first three samples, including the urines. However, the bladder tissue biopsies and the urine samples of the fourth patient yielded similar bacteria (Table 1). Both the urine specimens (TUC and SPA) had large amounts of Klebsiella pneumoniae and Staphylococcus aureus. A smaller amount of both organisms were cultured in the bladder biopsy tissue. I do not believe that this is an artifact of the urine because each tissue is washed twice with saline before being ground
for culture (as described in Methods and Materials). Other organisms were found in the bladder biopsy tissue but not in the urine specimens. This is most likely due to overgrowth of *K. pneumoniae* and *S. aureus*. Further work needs to be done to determine if these bacteria are truly associated with urothelial cells. If so, then one could investigate this interaction, its effect upon the urothelial cells, and whether any of those effects lead to OAB symptoms.

Culture of bladder biopsy tissue can provide an accurate picture of the bacteria that may be associated with the urothelium, but it is an invasive procedure and thus it can be a challenge to obtain samples. Therefore, to make more rapid scientific progress, it is important to use another method to identify the bacterial species in the urinary microbiota that associate with the urothelium. A less invasive approach is to examine shed urothelial cells in urine (Horsley *et al.*, 2013, Khasriya *et al.*, 2013).

Thus, I developed and attempted to optimize a protocol for future use to distinguish between the bacterial populations (planktonic, adherent, intracellular) of the urinary microbiota. The protocol combined three different assays. The first assay was filtration of the urine to distinguish between planktonic and associated bacteria (*Figure 2*), the second an antibiotic protection assay to distinguish between planktonic, adherent and intracellular bacteria (*Figure 3*), and the third was a tissue-culture adherence assay to aid in distinguishing between adherent and intracellular bacteria (*Figure 5*).

The antibiotic protection assay arm of the protocol was able to differentiate between the planktonic bacteria population and the bacterial populations that associate with the shed human urothelial cells. In the antibiotic protection assay arm, bacteria were
detected in some practice urine specimens in the no antibiotic treatment arm (Table 4, Table 6). All bacterial species detected in the urines were also detected in the EQUC control; however, they were detected at two-three logs lower CFU counts. These data suggest that the bacterial species in the no antibiotic treatment arm are associated with the shed urothelial cells and are not planktonic.

The antibiotic protection assay arm alone could not distinguish between bacteria that adhere to the urothelial cell surface and intracellular bacteria. In the proof of principle human urothelial cell/ UPEC infection assay, it was shown that the antibiotic protection assay could not detect intracellular UPEC in the immortalized human urothelial cells (Table 5). A modification to this protocol for the future would be to handle the cells with more care. In addition, it is unknown if adding trypsin in the proof of principle assay is harmful to the bacterial populations associated with the urothelial cells. In the control arm of the human urothelial cell/ UPEC infection assay, the cells were not treated with trypsin (Figure 4). Future control experiments would need to be performed to determine if trypsin has any effect.

However, this protocol was designed to use multiple assay/arms to obtain a more complete picture of the different bacterial communities that may associate with the shed urothelial cells. Some evidence that suggests the tissue-culture adherence arm could help further distinguish the associated bacterial population into bacteria that are adherent to the cell surface and intracellular bacteria. In the tissue-culture adherence assay, Dr. Meghan Pearce was able to detect several bacterial species in the antibiotic treatment branch that were not detected in the no-antibiotic treatment branch of two urine
specimens (Practice Urines 18 and 19) (Table 6). These data suggest that these bacterial species were intracellular.

Overall, these data demonstrate that these two assays complement one another. The results of the antibiotic protection assay show that it can distinguish between bacteria that are planktonic and bacteria that are associated with shed urothelial cells. The results of the tissue-culture adherence assay show that it can take the distinction one step further and thus distinguish between bacteria that may be adherent and bacteria that may be intracellular.
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VITA

Evann Hilt was born in Barrington, IL on November 28th, 1989 to Thomas and Ann Hilt. Before attending Loyola University Chicago, she attended Augustana College and graduated with a Bachelor of Arts with a double major in Biology and Pre-Medicine and minor in Biochemistry in May 2012.

In August of 2012, Evann joined the Infectious Disease and Immunology Research Institute at Loyola University Medical Center. She joined the laboratory of Dr. Alan J. Wolfe and was co-mentored by Dr. Wolfe and Dr. Paul C. Schreckenberger. While at Loyola, she studied the female urinary microbiota and its association with the human urothelium.

Once she has completed her Master’s of Science here at Loyola University Chicago, she plans to continue her education at Loyola to earn her PhD.