2012

Urinary Antimicrobial Peptides and the Urinary Microbiota in a UTI-Susceptible Population of Female Pelvic Floor Surgery Patients

Vanessa Nienhouse
Loyola University Chicago

Recommended Citation
https://ecommons.luc.edu/luc_theses/727

This Thesis is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Master's Theses by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.
Creative Commons License
This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2012 Vanessa Nienhouse
LOYOLA UNIVERSITY  CHICAGO

URINARY ANTIMICROBIAL PEPTIDES AND THE URINARY MICROBIOTA IN A
UTI-SUSCEPTIBLE POPULATION OF FEMALE PELVIC FLOOR SURGERY PATIENTS

A THESIS SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
MASTER OF SCIENCE

PROGRAM IN INFECTIOUS DISEASE AND IMMUNOLOGY

BY
VANESSA LAUREN NIENHOUSE

CHICAGO, ILLINOIS

MAY 2012
For Gracie.
ACKNOWLEDGEMENTS

I would like to thank my two basic science mentors, Dr. Alan Wolfe and Dr. Katherine Radek, and my clinical mentor, Dr. Linda Brubaker, for their unshakable support, encouragement and valuable guidance. Alan has provided much needed support during the endless trouble-shooting phases, but also was not afraid to push me and ask me to push myself to accomplish more than I ever thought I could. Katherine has been a source of endless creative and innovative ideas and I thank her for graciously taking me into her lab and envisioning this exciting project. Linda has served as an excellent role model and an invaluable window into the clinical world, helping me to truly make the most of the unique opportunities in this program. Additionally, I would like to thank my committee chair, Dr. Adam Driks, for his generosity with his time and advice.

I would not have accomplished any research without the support of every member of the Wolfe and Radek labs. Noriko Shibata and Steve Droho were instrumental in teaching me the basics of bench-work and I am extremely grateful for their patience and good humor. Other members of the Radek lab, Dr. Jennifer Plichta, Dr. Brenda Curtis and Tina Griffin have always been willing to lend a sympathetic ear, offer advice and provide expertise. Student meetings in the Wolfe lab
were some of the most productive times of my training, and I thank every student lab member for all their input.

Many thanks go to the Infectious Disease and Immunology Institute, its co-directors Dr. Katherine Knight and Dr. David Hecht, and the graduate program director, Dr. Karen Visick, for making every effort to help us get the most out of this graduate program and for valuing our feedback. Thank you also to Sara Hlavin and Michelle Laning, my fellow students/guinea pigs for your camaraderie and moral support.

Lastly, but arguably most importantly, I am eternally grateful to my family, Mommy, Daddy and Kyle; I could not ask for a more steadfast support system.
# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS**

**LIST OF FIGURES**

**LIST OF ABBREVIATIONS**

**ABSTRACT**

**CHAPTER ONE: INTRODUCTION – LITERATURE REVIEW**

- Clinical Impact of Urinary Tract Infection 1
- Host-Pathogen Interactions in the Urinary Tract 5
- Relevance of the Microbiota 7
- Antimicrobial Peptides 11
- Concluding Remarks 17

**CHAPTER TWO: MATERIALS AND METHODS**

- Sample Collection and Processing 18
- Enzyme-Linked Immunosorbent Assays (ELISA) 19
- High Pressure Liquid Chromatography (HPLC) 20
- Radial Diffusion Assay (RDA) 20
- Protease Assay 21
- 16S Sequencing and Bioinformatics 21
- Statistical Analyses 22

**CHAPTER THREE: EXPERIMENTAL RESULTS**

- Urinary Antimicrobial Peptide Levels 22
- Urinary Antimicrobial Peptide Activity 28
- Urinary Microbiota 36
- Integrated AMP and Microbiota Results 41

**CHAPTER FOUR: DISCUSSION**

- Sample Processing 44
- Urinary Antimicrobial Peptides 47
- Urinary Microbiota 50
- Integrated Analyses 54

**REFERENCE LIST**

**VITA** 66
LIST OF FIGURES

Figure

1. Protein assay comparison with the same samples reveals discrepancies between assays 25

2. Urinary antimicrobial peptides human β-defensin-1 and psoriasin exhibit great variability. 27

3. Antimicrobial peptides and urinary peptides elute at various acetonitrile concentrations during HPLC. 29

4. Baseline-positive urine tends to contain a greater variety and higher concentrations of protein and peptides than baseline-negative urine. 32

5. Baseline-positive urinary peptide fractions exhibit greater antimicrobial activity. 33

6. Urinary protease activity trends toward higher levels in baseline-positive urine than in baseline-negative urine. 35

7. Genetic diversity of urinary microbiota. 37

8. Microbiota profiles of postI-UTI and baseline-positive samples at the family level are similar. 39

9. Microbiota profiles postI-UTI samples at the genus level are similar. 40

10. Levels of psoriasin are lower in urine with detectable E. coli than in urine with other types of infection. 42
11. The number of sequences in the genera Streptococcus and Peptoniphilus each positively correlate with levels of psoriasin.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>°C</td>
<td>degrees celcius</td>
</tr>
<tr>
<td>AAD</td>
<td>antibiotic-associated diarrhea</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>AMP</td>
<td>antimicrobial peptide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment tool</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DAMP</td>
<td>danger-associated molecular pattern</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>h-BD</td>
<td>human beta-defensin</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-pressure liquid chromatography</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>sulfuric acid</td>
</tr>
<tr>
<td>IC/BPS</td>
<td>interstitial cystitis/bladder pain syndrome</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LL-37</td>
<td>cathelicidin</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>OTU</td>
<td>operational taxonomic unit</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>POP</td>
<td>pelvic organ prolapse</td>
</tr>
<tr>
<td>PostI-UTI</td>
<td>post-instrumentation urinary tract infection</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>RDA</td>
<td>radial diffusion assay</td>
</tr>
<tr>
<td>RDP</td>
<td>ribosomal database project</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>UI</td>
<td>urinary incontinence</td>
</tr>
<tr>
<td>UPEC</td>
<td>uropathogenic <em>E. coli</em></td>
</tr>
<tr>
<td>UTI</td>
<td>urinary tract infection</td>
</tr>
<tr>
<td>VDRE</td>
<td>vitamin D response element</td>
</tr>
</tbody>
</table>
ABSTRACT

Urinary tract infections (UTI)s are a national priority. Women who undergo surgery for pelvic floor disorders such as pelvic organ prolapse (POP) or urinary incontinence (UI) are at increased risk for UTI, as 10-30% will contract a post-instrumentation UTI (postI-UTI) within six weeks after surgery. Currently, the factors that contribute to the high rate of postI-UTI are unknown, and there is currently no clinical assessment to identify at-risk patients. However, antimicrobial peptides (AMPs) and characteristics of the urinary microbiota have the potential to serve as biomarkers, identifying patients at UTI risk and facilitating clinical prevention studies.

While it was previously thought that the urinary tract was a sterile environment, new evidence shows that bacteria inhabit the urinary tract in many people. Given that microbial communities (microbiota) in other areas of the body exist in a balance with the host defense system, a similar equilibrium likely exists between the microbiota and host defenses in the urinary tract. AMPs, one significant component of our innate defense system, can limit pathogenic infection by their abilities to interact with and disrupt microbial membranes, and to stimulate immune cell recruitment. While it is reported that certain AMPs are expressed in the urinary tract, the AMP profile of the urinary tract has not been characterized. Inappropriate expression of AMPs in other tissues, including altered levels and/or activity, has been associated with several
different disease states. It is therefore possible that POP/UI surgery patients that express inappropriate AMP levels or decreased AMP potency have an imbalance between their defenses and their resident microbiota, which results in their susceptibility to postl-UTI.

These results begin to reveal the urinary antimicrobial peptide and microbiota profiles of three cohorts of patients: (1) POP/UI patients with culture-negative urine samples at baseline who DO NOT develop a postl-UTI (2) POP/UI patients with culture-negative urine at baseline who DO develop a postl-UTI, and (3) POP/UI patients with positive clinical cultures at baseline. While levels of two AMPs, psoriasin and human β-defensin-1, do not significantly differ between the cohorts, preliminary evidence suggests that a characteristic microbiota may exist in patients who develop infection. Furthermore, AMPs and the microbiota may directly influence one another. Levels of psoriasin are lower in patients with detectable E. coli in their urine than in patients with other types of infections. In addition to this correlation, the presence of certain genera of bacteria positively correlates with levels of psoriasin. This could indicate that some members of the microbiota, by affecting levels of AMPs, influence the susceptibility of the urinary tract to invading pathogens. By furthering our understanding of urinary AMPs and the urinary microbiota, we take an important step toward being able to identify POP/UI patients who are at high risk for postl-UTI, and toward developing new therapeutic options for preventing infection.
CHAPTER ONE
INTRODUCTION - LITERATURE REVIEW

Clinical Impact of Urinary Tract Infections

Urinary tract infections (UTIs) are an important and costly clinical problem. Different reports suggest that thirty to sixty percent of adult women will experience a symptomatic UTI during their lifetime [1, 2]. In the US, it is estimated that there are over eight million episodes of UTI per year [3], and the annual cost associated with the treatment and care of UTIs is thought to exceed $2 billion [4]. Infections of the lower urinary tract, where they are termed cystitis, are associated with symptoms of dysuria, frequency, urgency, and lower abdominal or suprapubic pain [2]. Infections of the lower urinary tract can ascend to the kidneys causing pyelonephritis with symptoms of fever, costovertebral tenderness, nausea and vomiting [2]. Pyelonephritis can result in renal scarring and renal failure and can progress to systemic infection and even death [1].

UTIs are considered uncomplicated if they occur in otherwise healthy non-pregnant women. UTIs are considered complicated when they occur in patients with additional morbidity factors that could contribute to the failure of therapy, such as structural or functional abnormalities of the urinary tract, history of renal stones, use of catheters, infection with antibiotic-resistant organisms or being immunocompromised [2, 3]. Using conventional clinical urine culture techniques to identify bacteria, the most
common uropathogen is *E. coli*, which accounts for 80-85% of UTIs in premenopausal women. In postmenopausal women and in women with complicated UTIs, other uropathogens, such as *K. pneumoniae, Staphylococcus saprophyticus, Proteus, Pseudomonas, Enterobacter, Serratia* and other Gram-negative enteric bacteria, are common [2]. Many of these organisms frequently colonize the gastrointestinal tract and infection of the urinary tract is thought to be preceded by colonization of the vagina and then the periurethral area. The vaginal bacterial community plays a role in susceptibility to UTI. For example, hydrogen-peroxide producing strains of lactobacillus that normally inhabit the vagina inhibit the colonization of uropathogens and thereby help prevent their transmittance from the gastrointestinal tract to the urinary tract [3].

Risk factors associated with higher rates of UTI include recent sexual intercourse, use of spermicide-associated contraceptive methods [5] and possibly a shorter distance between the urethra and the anus [1]. In addition, the association of a number of genes with susceptibility to recurrent UTI suggests a possible genetic predisposition for risk of infection. These genes encode CXCR1 (the alpha subunit of the IL-8 receptor) [6], CXCR2 (the beta subunit of the IL-8 receptor), TLR2, TLR4 and TGF-β1 (a cytokine with multiple downstream effectors) [2]. All of these genes mediate either adaptive or innate immune responses.

Patients undergoing corrective surgery for pelvic organ prolapse (POP) and/or urinary incontinence (UI) are at increased risk for UTI. These surgery patients represent a significant population; one study estimated that up to 50% of vaginally parous women
lose pelvic floor support, resulting in prolapse, and found that the likelihood of a woman undergoing a POP/UI procedure is 11.1% [7]. Reported rates of post-instrumentation UTI (postI-UTI) vary depending on the definition used for UTI diagnosis. One study reported a postI-UTI rate of 5.9% in UI patients undergoing midurethral sling procedures, defining UTI as symptoms of UTI with a urine culture containing ≥50,000 cfu/mL of a single bacterial species within one month after surgery [8]. Another report found a postI-UTI rate of 9% in POP/UI surgery patients, defining UTI as irritative voiding symptoms accompanied by a positive urine culture within six weeks after surgery [9]. Finally, the SISTEr trial, which compared Burch colposuspension with autologous fascial sling for the treatment of stress incontinence, and defined UTI as culture-proven bladder infection or clinical suspicion of bladder infection resulting in treatment, found the rate of postI-UTI to be 48% in patients undergoing the sling procedure and 32% in the Burch procedure patients within twenty-four months after surgery [10]. Regardless of the definition used, postI-UTI rates are still strikingly high.

PostI-UTIs remain a problem despite preventive actions, such as the use of standard sterile technique during surgery, the administration of prophylactic antibiotics at the time of surgery, and several defensive mechanisms employed by the female urinary tract to prevent infection. These defensive mechanisms include shedding of uroepithelial cells with adherent microorganisms, secretion of mucus from the paraurethral glands to trap bacteria, and the intermittent wash-out of the urethra and bladder by the flow of urine [11]. Given all of these defensive and preventive
mechanisms, it is currently not known why the prevalence of postI-UTI is so high. Some patient characteristics have been identified as risk factors, such as elevated preoperative postvoid residual volume [8], but no biomarkers have been identified that could serve to identify patients at high risk for postI-UTI.

Although the high rate of postI-UTIs in this population of POP/UI surgery patients is a clinical problem, their well-documented clinical characteristics and their known risk make them ideal subjects for the study of UTIs. By recruiting POP/UI patients and obtaining urine specimens before the time of surgery and thus before infection, we have a unique opportunity to identify differences in the baseline status of patients who ultimately come down with infection. Identifying who is at risk for infection will be an important first step in understanding why these patients are at risk, and eventually lead to the advent of better means of preventing infection.

It is likely that no single characteristic can be used as an identifying risk factor, but rather that a number of factors play a role in indicating who is at risk. Urinary tract infections are the result of dynamic interplay between host factors like the innate immune system, pathogen factors like the expression of adhesins that aid bacteria in attachment and invasion [12], and factors pertaining to the environment of the genitourinary tract, such as the presence or absence of commensal bacterial species in or around the urinary tract. We will need to study each of these factors in the context of the others to fully understand the factors that contribute to risk for postI-UTI.
Host-Pathogen Interactions in the Urinary Tract

As described above, urinary tract infections are likely the outcome of dynamic interactions between the host immune system, the pathogens, and other members of the microbiota. Illustrative of these dynamics are several interactions that have been elucidated between known uropathogens and the human urinary tract. One of the first putative mechanisms of host defense that a pathogen will encounter in the urinary tract is the outward flow of urine, making the ability to adhere to the uroepithelium an important virulence factor. Uropathogens express a variety of adhesion molecules, e.g. MR/P fimbriae expressed by *Proteus mirabilis* and Type-1 Pili expressed by uropathogenic *E. coli* (UPEC) [6]. Type-1 pili bind uroplakin, a structural protein expressed on the surfaces of uroepithelial cells, via the FimH adhesin located at the tip of the pilus [12]. Both of these uropathogens also secrete toxins that contribute to disease, express flagella that aid in motility and ascension of infection, and possess iron acquisition systems [6].

The adhesive interaction of UPEC not only allows it to adhere to the mucosa, but also results in the stimulation of various host defense responses. Upon binding by FimH, superficial uroepithelial cells, which normally have a very slow turnover rate, are stimulated to rapidly exfoliate and to be shed in the urine, taking any adherent pathogens with them [13]. Additional innate defense factors include the typically low pH and osmolarity of urine, which are not conducive for bacterial growth, and lactoferrin in urine, which scavenges the iron necessary for bacterial growth. Other soluble defense
factors include Tamm-Horsfall protein, low molecular weight sugars and secretory IgA, all of which can bind to bacteria and inhibit their ability to adhere to the epithelial surface [12, 14]. The FimH interaction with epithelial cell receptors, augmented by TLR4 detection of LPS, also induces uroepithelial cells to produce IL-6 and IL-8, which subsequently activate a number of inflammatory pathways and recruit neutrophils, respectively [12].

UPEC have developed mechanisms to evade these host defenses. Compared to non-uropathogenic E. coli, UPEC elicit a less robust inflammatory response by avoiding detection by pattern-recognition receptors and inhibiting the NF-κB mediated expression of AMPs, pro-inflammatory cytokines and chemokines [15, 16]. The resulting delay in neutrophil recruitment allows UPEC to gain an early foothold, invade epithelial cells, and actually utilize the relative safety of the interior of those cells as an environment to replicate and persist in biofilm-like communities out of which they may periodically emerge. Upon emergence from the intracellular environment, a phenomenon termed “fluxing,” some of the cells adopt a filamentous morphology that allows them to avoid neutrophil phagocytosis and then reattach to the epithelium [17, 18]. Thus, these intracellular bacterial communities may serve as reservoirs for recurrent infection.

In recent years, our understanding of the interactions between the host and the pathogen during urinary tract infection has vastly expanded. However, there is a gap between the body of research knowledge and the realm of clinical practice. Clinical
interventions for urinary tract infection have essentially not changed for decades. With
the increasing prevalence of antibiotic-resistant organisms, and in light of the
knowledge that some bacteria in the urinary tract could escape the action of antibiotics
by harboring in epithelial cells, there is a great need for the development of new
therapeutic practices. These advances should take into account all of the knowledge
amassed in the research community about patients who are most at risk for infection,
and should aim to develop prevention methods that could specifically target these
groups. Such advances will likely manipulate different aspects of the interactions
between the host, the pathogen and the environment of the urinary tract.

Relevance of the Microbiota

Communities of microorganisms are being characterized in several body
systems, including the oral cavity, the vagina and the gastrointestinal tract [19]. The
Human Microbiome Project aims to discover the diversity of the microbiota associated
with humans and to determine if there is a common microbiota profile, termed a “core”
microbiota, that is prolific in the population and indicative of health. There is some
evidence that such a core microbiota may exist in the oral cavity, whereas in the vagina
and gastrointestinal tract, much more variation is observed, and there is no obvious
single microbiota profile associated with a healthy state [19].

Nevertheless, there is a plentitude of evidence indicating that our microbiota are
essential for our health. The microbiota in the gastrointestinal tract are particularly well-
studied. Knowledge of this community provides considerable insight into the complex
interactions between the host and the resident microbiota and reveals a truly symbiotic relationship. Commensal bacteria in the gut can aid in the production and digestion of nutrients, allowing hosts to exploit nutrient sources they would otherwise be unable to utilize [20]. For example, many fermenting bacteria can utilize a wide range of polysaccharides to produce butyrate, a molecule that serves as a major energy source for colonic cells [21]. In studies performed with germ-free mice, it has been shown that establishing and maintaining a microbial community in the gastrointestinal tract is essential for proper immune system development and function [22]; the microbiota modulate the activity of dendritic cells [23] and T-cells [24] and can contribute to proper immune balance through the induction of anti-inflammatory effectors [25]. Interaction of the microbiota of the GI tract with the host mucosa provides protection against intestinal damage by inducing expression of different genes through TLR signaling [26]. There is also evidence that the presence of particular microbes in the flora may impart some protection against pathogenic bacteria [27] either by competitively binding and inhibiting their adherence [28], competing for nutrients [29] or by inhibiting the proliferation of the pathogen through the production of antimicrobial compounds [19].

Similarly, in the vagina, Lactobacillus is often the predominating genus, and is thought to inhibit the growth of invasive bacteria through the production of hydrogen peroxide, by maintaining a low vaginal pH through the production of lactic acid, and by competitively filling an ecological niche [30]. However, this equilibrium can be disturbed
when Lactobacillus loses its prominence, and is instead replaced by predominantly anaerobic bacteria. Women with this bacterial profile are much more susceptible to a number of urogenital diseases, including bacterial vaginosis, yeast infections, sexually transmitted infections including HIV [31] and *Neisseria gonorrhoeae* [32], and urinary tract infections [30].

The example of Lactobacillus in the vagina demonstrates that the microbiota can have a profound influence on the host. The opposite also is true: host factors can play an integral role in shaping the microbial community [33]. For example, host genetics can play a role in microbial diversity. In one study, decreased diversity was reported to correlate with a mutation in a gene associated with familial Mediterranean fever [34]. Furthermore, gut microbes are controlled by diverse immune effectors, such as the adaptive immune effector secretory IgA, and the innate immune effectors antimicrobial peptides [35, 36].

An imbalance in the dynamic equilibrium between the host and microbiota can lead to disease. In many cases, it is not clear if the disruption in the bacterial community precedes or results from disease and whether the microbiota disruption is the cause. However, in some cases, the causative relationship is fairly clear. Antibiotic use profoundly perturbs the intestinal microbiota composition, interfering with many important interactions between members of the bacterial community and with the host, often resulting in antibiotic-associated diarrhea (AAD) [37]. In many cases, this perturbation allows an opportunistic pathogen to gain a foothold and cause disease. The
most common example of this is *Clostridium difficile*, the leading cause of pathogen-associated AAD [38]. This association between disruption of the microbiota and *Clostridium difficile* infection gives rise to the idea that restoring the intestinal microbiota to its pre-antibiotic status could be beneficial in reestablishing healthy interactions, a concept supported by the effective treatment of recurrent *C. difficile* infections by inoculating patients’ GI tracts with donor stool specimens [39].

Although much is known about the dynamics of the microbiota at several body sites like the GI tract, little is known about the microbiota found in the urinary tract. Current dogma states that the urinary tract and the urine within are sterile [40]. This dogma arose, largely because clinical practice is to identify infecting organisms via culture techniques, which do not grow organisms in the absence of infection. However, this concept of sterile urine could merely be due to the fact that a vast majority of bacteria are not culturable using these standard laboratory techniques [19]. Even normally culturable organisms can enter a state in which they are viable, but not culturable [41], and bacteria in this state have been documented in the urine [42]. The culture method provides incomplete and skewed information about the bacteria in any sample. On the other hand, molecular methods, in use for decades as a means of identifying bacteria in the environment [19], provide much more depth and thus a much more complete picture of the bacterial community in any sample.

In fact, recent studies offer culture-independent evidence of bacteria in both the male [43, 44] and female urinary tracts [45, 46]. DNA sequencing technology allows us
to detect and identify bacteria based on the presence of 16S rRNA genes even in culture-negative urine. Given the evidence that interactions between members of the microbiota and the host play a significant role in health and disease [47], the discovery of bacteria in the urinary tracts suggests the likelihood that a similar dynamic environment exists there. Supporting this idea is evidence that, in males, certain urinary microbiota profiles dominated by fastidious, anaerobic and uncultivated bacteria can be associated with the presence of sexually transmitted infections [44]. Interstitial cystitis/bladder pain syndrome (IC/BPS) is a urinary tract disorder with unknown etiology. Some studies report an association of low-count microorganisms with IC/BPS [48], whereas other studies exclude the possibility of an infectious etiology for IC/BPS [49]. However, all of these studies utilized culture-techniques. Culture-independent methods might bring clarity to the question of whether bacteria are responsible for IC/BPS. It is conceivable that urinary tract diseases with previously unknown etiologies may be caused by disruptions in the balance between the normal microbiota and the host’s defenses.

**Antimicrobial Peptides**

Antimicrobial peptides (AMPs) are small (i.e. ~10-40 amino acids) cationic, amphipathic peptides that serve as an integral component of our innate defense system. They exhibit broad-range killing activity against Gram-negative and Gram-positive bacteria, fungi, protozoa, and enveloped viruses [50]. Several AMPs are expressed by humans. Some of the best studied are alpha- and beta-defensins,
cathelicidin (LL-37), RNase 7, hepcidin, cathelicidin and psoriasin (S100A7). The observation that organisms throughout the plant and animal kingdoms produce AMPs suggests that they are evolutionarily ancient and serve an essential role in the defense of many species from infection. In humans and other mammals, they are expressed by epithelial cells on the skin and at other mucosal surfaces, as well as in granules of phagocytic cells [51]. Different classes of AMPs can work together synergistically to provide additional antimicrobial activity [52]. As such, they constitute one of our first lines of defense against infection. AMPs can recognize and destroy invading pathogens even before an inflammatory response is mounted and before innate immune cells, such as neutrophils, are recruited to the site of infection. Furthermore, they protect against invasive pathogens long before the adaptive immune system can mount an antigen-specific response [51, 53-55].

The cationic and amphipathic properties of these molecules are important because they allow AMPs to recognize and interact with microbial membranes, cause membrane disruption, and thus ultimately kill the target microbe. Positively charged AMPs can distinguish microbial membranes from host cell membranes because the former generally contain phospholipids with negative head groups in the outer membrane leaflet, while the latter contain phospholipids with no net charge [51, 55].

There are a few proposed models for the mechanism of membrane disruption. The Shai-Matsuzaki-Huang models describe two potential mechanisms. In the barrel-stave mechanism, low numbers of peptide interact with a target membrane via
electrostatic interactions and polymerize to form a pore in the membrane, causing the cell to lose membrane potential and allowing contents of the cell to leak out. The carpet mechanism states that the peptides, when present at high enough concentrations, will spread out across the surface of the membrane causing a loss of membrane curvature and leading to the formation of transient toroidal pores [56-58]. Supporting these concepts is evidence that the hydrophobic portion of LL-37 inserts into the core of a lipid bilayer altering the capacity of the lipids to pack together properly [59]. In any of these cases, the integrity of the cellular contents is compromised allowing for rapid lysis of the target cell [51, 55, 60]. There is additional evidence that once the membrane is permeabilized, peptides can enter the cell and associate with intracellular targets, inhibiting cell-wall, nucleic acid or protein synthesis or inhibiting enzymatic activity [60].

More recently, it has been reported that AMPs also serve as important signals for the immune system, recruiting immune cells to sites of inflammation and helping to enhance host immune activity by inducing the expression of pro-inflammatory cytokines and chemoattractant molecules [50, 55]. Cathelicidin, in particular, acts as a chemokine for neutrophils, monocytes, mast cells and T-cells [61], and can affect transcription of cytokines by macrophages and keratinocytes [62]. Additionally, it plays an important role in wound healing, as it stimulates re-epithelialization of skin wounds [63] and promotes angiogenesis [64]. Human α-defensins can chemoattract T-cells and induce the release of IL-8 from epithelial cells [65]. β-defensin-3 (hBD-3) stimulates monocytes to produce the important inflammatory response mediators, IL-6, IL-8 and IL-1β [66]. In
human oral epithelial cells, β-defensins stimulate the secretion of the lymphocyte chemoattractant MIP-3α [66]. Recently, it was shown that β-defensin-1 (hBD-1) induces neutrophils to form neutrophil extracellular traps (NETs), webs of DNA that trap and kill pathogens [67]. Thus, AMPs induce a variety of immune processes and play additional roles in host defense apart from their directly antibacterial activities.

Much is also known about the signals that lead to AMP expression. Production of AMPs can be constitutive, or production can be induced by various stimuli. For example, hBD-1 is constitutively expressed [68-71]. In contrast, cathelicidin, β-defensin-2 (hBD-2) and psoriasin are each induced by the activation of host cell receptors that recognize danger-associated molecular patterns (DAMPs), including pathogen-associated molecular patterns (PAMPs) [61, 68, 70-72]. For example, hBD-2 expression is induced by TLR-2 and TLR-4 activation in intestinal epithelial cells [73]. Flagellin-stimulated activation of TLR-5 induces the expression of hBD-2 and psoriasin in epidermal keratinocytes [72]. Activation of TLRs results in an intracellular signaling cascade, leading to the activation and nuclear translocation of NF-κB. This transcription factor up-regulates transcription of various genes, including many AMPs and proteins involved in AMP-dependent responses [50]. Expression of these inducible AMPs also responds to cytokine stimulation and other signals. In intestinal epithelial cells, hBD-2 expression is induced by IL-1α. In monocytes, both hBD-2 and cathelicidin are induced by IFN-γ [74]. In addition, the promoter for the cathelicidin gene contains a vitamin D response element (VDRE) and expression of this peptide is strongly induced by the active form of
vitamin D, 1,25-dihydroxyvitamin D₃ [75, 76]. Vitamin D-dependent signaling also serves as a pathway through which cathelicidin and TLR-2 expression can be upregulated in response to injury [77]. Thus, AMPS can be induced by invading pathogens, or by other signs of stress, such as inflammation or injury.

AMPs affect the composition of the resident microbiota, as discussed above, but microbes have developed mechanisms for influencing the function or expression of host AMPs for their own benefit. One example of a resistance mechanism against AMPs is found in Proteus mirabilis, a major uropathogen as previously discussed. This organism secretes a metalloprotease, ZapA, with a wide range of targets including AMPs. Secretion of ZapA allows P. mirabilis to degrade and thus escape several immune elements [78]. On the other hand, the commensal organism Staphylococcus epidermidis actually induces the expression of AMPs, including hBD-2 and 3, in keratinocytes on the skin via a TLR-2-dependent mechanism. This interaction is beneficial for both the host and microbe, because the AMPs kill pathogens on the skin, protecting the host from infection, and allowing S. epidermidis to proliferate with fewer competitors for resources [79].

Nearly all AMPs are synthesized as pro-proteins and undergo a series of proteolytic processing steps before reaching their bioactive form. Cathelicidin's proform, for example, consists of two domains, the C-terminal domain which, when the peptide is cleaved, goes on to become the mature antimicrobial peptide, and the N-terminal “cathelin-like” domain. Until recently, it was not known that this cathelin-like
domain actually exerts effects of its own; it exhibits antimicrobial activity against *E. coli* and methicillin-resistant *S. aureus*, and additionally displays protease inhibitory activity, possibly serving to mitigate tissue damage during inflammation [80]. The proteases responsible for cleaving cathelicidin into its mature form are the serine proteases kallikreins 5 and 7. These enzymes are also responsible for further processing mature cathelicidin into shorter peptides with various antimicrobial and immune signaling activities [81]. The expression of these kallikreins in keratinocytes is upregulated by calcium, vitamin D$_3$, and by retinoic acid [82]. Alpha-defensins are detectable as a 7kD prodefensin in granulocytic cells and macrophages before they are processed to their mature 3.5kD form, although the enzymes responsible for this processing are not known [71].

There is much still to learn about the role of antimicrobial peptides in the urinary tract. Their importance in this system is shown by knock-out animal models. Both cathelicidin-deficient (*Camp*$^{-/-}$) mice and β-defensin-1 deficient (*Defb1*$^{-/-}$) mice are more susceptible to UTI than their wild-type counterparts. In humans, hBD-1 is constitutively expressed by epithelial cells in the kidney [69], while hBD-2 is induced in these cells by infections [83]. Other AMPs that have been identified in the urinary tract include α-defensins derived from neutrophils, and hepcidin which serves dual roles. It is directly antimicrobial, and it also regulates iron availability, reducing the amount available for invading microbes [83]. However, the specific roles these peptides play in disease and infection of the urinary tract has not been entirely elucidated.
Concluding Remarks

Given all that we know about the dynamic interactions between the host, the commensal members of the microbial flora and the pathogens in various body systems, I hypothesize that careful analysis of interactions between these diverse players in the urinary tract will yield fruitful information about disease and infection. Analyzing urinary AMPs, a factor of host defense, and the urinary microbiota in our population of POP/UI surgery patients may reveal that certain characteristics in these profiles are indicative of risk for post-UTI. Identifying who is at risk will be an important step toward being able to prevent these infections through improved therapeutic intervention.
CHAPTER TWO

MATERIALS AND METHODS

Sample Collection and Processing

We collected transurethral catheterization (TUC) urine samples from POP/UI surgery patients at the time of surgery under an IRB approved protocol. Samples were held on ice no longer than 4 h before distribution in 3 parts. One fraction was sent to the Loyola University Medical Center clinical microbiology laboratory, which determined the culture status. One fraction was processed for DNA sequencing by the addition of a DNA stabilizing agent. This fraction was then frozen at -80°C until it was shipped to the Nelson laboratory at Indiana University-Bloomington, where it was sequenced. A third fraction was centrifuged at 13,300 rpm for 20 minutes and the supernatant was filtered through a .22um filter to remove particulate matter and cell debris. To solubilize peptides in the urine and to inhibit any proteases that may be present, 10% trifluoroacetic acid (TFA) was added to each sample to acidify it by reducing the pH to approximately three, which yields a final 0.1-0.5% TFA concentration. After the initial processing steps, the samples were aliquoted and stored at -80°C until further analysis steps.
To measure the concentration of each AMP, I used ELISA kits for human β-defensin-1 (PeproTech, Cat # 900-K202) and psoriasin (Circulex, Cat # CY-8073) and followed the manufacturers’ instructions.

For the hBD-1 kit, a 96-well plate was incubated with a capture antibody overnight and then blocked with 1% BSA for one hour. Samples and standards were incubated for two hours and then concentrations of the target peptide were measured with a biotinylated detection antibody incubated for two hours, which was itself detected with an avidin-HRP conjugate incubated for 30 minutes, and its substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (green) incubated for approximately 45 minutes. Absorbances were monitored at five minute intervals with a plate reader at 405 nm with wavelength correction set at 650 nm until the absorbance for the highest standard at 405 nm reached an OD of 1.2.

For the psoriasin kit, samples and standards were incubated for one hour in a 96-well plate that had been pre-coated with capture antibody. An HRP-conjugated detection antibody was added and incubated for one hour. The substrate was then added and the plate was allowed to develop for 10-20 minutes before 1N H₂SO₄ was added to stop the reaction. Absorbance was measured with a plate reader at 450nm with wavelength correction set at 540 nm.
Values were normalized to the total protein in each sample as measured by a Bradford protein assay (Bio-rad, Cat # 500-0006). Dye-reagent was added to the samples at a 1:5 ratio and mixed well. Absorbance was measure with a plate reader at 595 nm.

**High Pressure Liquid Chromatography (HPLC)**

Using a Shimadzu HPLC machine, samples were injected onto a C18 column (Thermo Scientific, Cat # 25008-254630) in 90% water, 10% acetonitrile (ACN) mobile phase buffer. Over the course of 50 minutes, the concentration of ACN was increased to 90%. As the concentration increased, increasingly hydrophobic peptides eluted off the column. A UV detector at 214 nm measured the peptide bonds, and a UV detector at 280 nm measured aromatic side chains of amino acids. Fractions of eluate were collected at one-minute intervals between 10% and 55% ACN. To ensure each fraction contained enough peptide for further analysis, three 1.5 mL aliquots were run separately, and the fractions from each run were combined.

**Radial Diffusion Assay (RDA)**

Corresponding fractions from each sample were lyophilized to remove residual acetonitrile, reconstituted in sterile water, and vortexed for one hour at 4°C to fully dissolve the peptides. The peptide concentration of each fraction was measured by a spectrophotometer at 214 nm. Then, more sterile water was added, such that corresponding fractions from each sample contained the same peptide concentration. For the agar plate, 100 µL of a $10^8$ CFU/mL culture of ΔmprF S. aureus were added per 25 mL of 0.75% Top agar, and 10 mLs of agar were poured in a plate and allowed to
solidify. Wells were made in the agar with vacuum suction through a sterile Pasteur pipette. One microliter of each HPLC collected fraction was added to individual wells. Positive controls were 100 µM synthetic catestatin and 64 µM synthetic cathelicidin, and water was used as a negative control. The plates were incubated overnight at 37°C. The area of each zone of inhibition was measured using ImageJ software.

**Protease Assay**

To assess protease activity, an Enzchek protease assay (Invitrogen, Cat # E6638) was used, following manufacturer’s instructions. Briefly, samples were incubated with BODIPY-FL, a macromolecule containing multiple fluorescent markers that are quenched when the molecule is intact. Upon proteolytic cleavage, a green fluorescent signal was released. Fluorescence was measured with a microplate reader with excitation and emission filters of 485 nm and 530 nm, respectively.

**16S Sequencing and Bioinformatics**

Bacterial DNA sequencing and bioinformatics to determine the identity of microbes present in urine samples were performed by our collaborators Dave Nelson and Qunfeng Dong, respectively, as described [45]. Briefly, the V1-V3 regions of the bacterial 16S rRNA gene were PCR-amplified, bar-coded and sequenced via pyrosequencing using the Roche 454 Titanium platform. The resulting sequences were analyzed using a BLAST search to identify and discard any sequences with too great a homology to human genes, and the remaining sequences were assigned taxonomic classifications using RDP Classifier v.2.2.
Statistical Analyses

To facilitate integrated analyses of several different types of data, I have constructed a database that includes clinical information about each patient, the patients’ responses to a questionnaire that describes the severity of their symptoms, the levels of AMPs in their urine and data about their microbiomes, including culture status and sequencing data. With the help and direction of Qunfeng Dong, our bioinformatician collaborator, we clustered patients based on similarities of their microbiota using a Spearman’s rank correlation coefficient as a distance measure. A series of univariate and multivariate linear regressions were performed to identify correlations between levels of AMPs and the abundance of certain microbes.
CHAPTER THREE

EXPERIMENTAL RESULTS

Urinary Antimicrobial Peptide Levels

A subset of POP/UI surgery patients will contract a postI-UTI within six weeks of surgery. Currently, before surgery, there is no way to identify patients who are at risk for a postI-UTI. Because AMPs are antimicrobial agents, we hypothesize that high or low levels of one or more urinary AMPs may indicate risk for development of a postI-UTI.

We therefore compared the AMP levels in the following three cohorts:

1) **Baseline-positive**, i.e. patients with culture-positive urine on the day of surgery (n = 13)

2) **Baseline-negative**, i.e. patients with culture-negative urine on the day of surgery who **DO NOT** develop postI-UTI (n = 122)

3) **PostI-UTI**, i.e. patients with culture-negative urine on the day of surgery who **DO** develop a postI-UTI (n = 8)

Before we could perform this comparison, however, we had to normalize the urinary AMP concentrations to account for dilution of the urine due to differences in patient solid and liquid consumption. One valid approach is to normalize urinary AMP levels to the total protein content of the urine. Through trial and error, we discovered that some components in the urine (e.g., creatinine, uric acid or various amino acids)
might interfere with the mechanisms of some of the more commonly used protein assays and thus lead to false positive results. For example, agents with reducing potential, chelating agents, and strong acids or bases are known to interfere with BCA assays and Lowry assays, because their method of protein detection involves the chelation of a metal ion.

To identify the protein detection method most appropriate for urine, we measured six samples with three different assays: a Bradford assay, a modified Lowry assay, and a BCA assay. The values from each assay drastically differed even when using the same BSA standard concentrations for each assay (Figure 1). Because no two assays produced similar results, we were unsure which method yielded the most accurate values. We therefore submitted the samples to the clinical core lab at Loyola. They measured the protein content of each sample on the Beckman Coulter Synchron DxC800 system, which uses pyrogallol red and molybdate to form a purple color complex with protein that is detectable by measuring absorbance at 600 nm. For each sample tested, the results were reported as <60ug/mL, which is their lower limit of detection (data not shown). These results agreed most closely with the results obtained with the Bradford assay, which by far gave the lowest values. Thus, we concluded that the Bradford assay was the most accurate method to measure urinary protein and for the remainder of this study, we used the Bradford assay to normalize all of the AMP concentrations.

To measure the concentrations of the known urinary AMPs, hBD-1 and psoriasin, I performed ELISAs. The hBD-1 concentrations varied considerably within each cohort.
Figure 1. Protein assay comparison with same samples reveals discrepancies between assays. (A) The same urine samples tested with three protein assays: a Bradford assay, a modified Lowry assay and a BCA assay. (B) Results from the Bradford assay alone.
and no significant differences were detected (Figure 2a). Between cohorts, the average levels of hBD-1 and the distributions of those levels were very similar, with a vast majority of the samples exhibiting minimal levels and fewer samples exhibiting greater levels of hBD-1. While the concentrations of psoriasin were much lower than those of hBD-1 (Figure 2b), the levels of psoriasin generally followed a similar pattern, with the majority of the samples exhibiting very low levels and a small proportion displaying higher levels. In contrast to those of hBD-1, however, psoriasin levels tended to be higher in baseline-positive urine than in the baseline-negative urine, although the differences were not significant. Moreover, baseline-positive psoriasin levels demonstrated a bimodal distribution. A larger subset of patients in the culture-positive cohort expressed relatively high levels of psoriasin, whereas the rest expressed minimal levels. Urinary psoriasin was minimal in all patients who were culture-negative at baseline, but later contracted a postI-UTI.
Figure 2. Urinary antimicrobial peptides, human β-defensin-1 and psoriasin, exhibit great variability. Concentrations of hBD-1 (A) and psoriasin (B) in urine samples from patients in three cohorts as measured by ELISA and normalized to total protein levels. Data points represent mean values of two independent ELISAs performed with each sample.
Urinary Antimicrobial Peptide Activity

HBD-1 and psoriasin are just two of the many AMPs that could be present in urine. Furthermore, the amount of AMPs present in the urine does not necessarily relate directly to their activity. Mutations in the genes that encode AMPs or the genes that encode the proteins responsible for correctly processing AMPs can cause different individuals to express AMPs with different functional capacities or, in other words, different antimicrobial activities. By comparing the functional capacities of AMPs in urine samples obtained from patients from all three patient cohorts, we may reveal that higher or lower levels of antimicrobial activity are indicative of risk for post-UTI. To assess the antimicrobial activity of AMPs in each urine sample, we fractionated the peptides within a sample by HPLC and then performed a solid-phase bacterial-growth-inhibition assay, the radial diffusion assay.

To determine when we could expect AMPs to elute off the C18 column during HPLC, we ran through the column three solutions of known AMPs. As expected, because of their amphipathic nature, the three AMPs, psoriasin, hBD-1 and cathelicidin, eluted off the column at an intermediate concentration of acetonitrile in the mobile phase buffer, between 40% and 55% acetonitrile (Figure 3a-c). Amphipathic peptides from a urine sample eluted between 10% and 55% acetonitrile; therefore, to separate the peptides contained in one sample, fractions were collected at one minute intervals between these concentrations, which corresponds to minutes 10-35 during one fractionation run (Figure 3d).
Figure 3. Antimicrobial peptides and urinary peptides elute at various acetonitrile concentrations during HPLC. (A-C) Three known AMPs elute between 40% and 55% concentration of acetonitrile in water. (D) Urinary AMPs elute between 10% and 55% concentration of acetonitrile in water. Shaded bars indicate one-minute intervals in which fractions of eluate are collected.
I compared the tracings between samples from each of the three cohorts. Although not universally the case, the HPLC tracings of baseline-positive urine samples tended to show larger and more frequent peaks than baseline-negative urine (Figure 4), indicating that, in general, culture-positive samples contain a greater variety and higher concentrations of peptides and proteins.

The next step in determining the functional capacity (i.e., antimicrobial activity) of urinary AMPs was to subject each HPLC fraction to a radial diffusion assay. This assay, which measures antimicrobial activity through bacterial growth inhibition, was performed with fractions of a subset of urine samples from each cohort. Antimicrobial activity was tested against a ΔmprF Staphylococcus aureus strain. Multiple peptide resistance factor (mprF) is a bacterial membrane protein that regulates membrane charge by synthesizing and transferring positively charged lysyl-phosphatidylglycerol to the outer leaflet of the cell membrane. Strains with mprF mutations have more negatively charged membranes making them more susceptible to cationic AMPs [84]. We use this strain, because its susceptibility to AMPs makes it easier to observe differences in antimicrobial activity.

Urinary peptide fractions from baseline-positive samples tended to exhibit greater frequency and magnitude of antimicrobial activity against ΔmprF S. aureus than peptide fractions from baseline-negative urine (Figure 5); however, in all three cohorts there were samples that did not exhibit antimicrobial activity against this strain in any fractions. In this regard, there are no obvious differences between the cohort of
baseline-negative patients who develop a postI-UTI (postI-UTI positive) and the cohort of baseline-negative patients who do not. Of the samples that exhibited antimicrobial activity, some trends are apparent. For example, certain fractions more frequently exhibited antimicrobial activity (e.g., fractions 14-16, 24 and 33-34).
Figure 4. Baseline-positive urine tends to contain a greater variety and higher concentrations of protein and peptides than baseline-negative urine. (A) HPLC tracings of two baseline-positive urine samples. (B) HPLC tracings of two baseline-negative samples, one from a patient who contracted a postI-UTI, and one from a patient who did not. Tracings are representative of their corresponding cohort, although not all samples in one cohort always followed the trend.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
<th>29</th>
<th>30</th>
<th>31</th>
<th>32</th>
<th>33</th>
<th>34</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Culture Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa - 1055</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Enterococcus - 585</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. coli - 817</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Staph aureus, Corynebacterium - 868</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. coli - 1081</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. coli - 1018</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. coli - 690</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. coli, K. pneumoniae - 814</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa - 916</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa - 1015</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. coli - 421</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. coli - 537</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Post-UTI Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa - 564</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Enterococcus - 661</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Klebsiella oxytoca - 1049</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. coli - 830</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Enterococcus - 489</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>M. morganii, S. maltophilia - 552</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E. coli - 835</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Baseline Culture Negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>808</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>844</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1075</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1027</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>684</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>687</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>699</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>805</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>994</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1039</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1127</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
AMPs undergo various stages of proteolytic processing. They are synthesized as pro-proteins, which are then cleaved into their bioactive form. Further cleavage degrades them. Because proteases are responsible for the processing of AMPs to their mature form, antimicrobial activity is integrally associated with protease activity. Measuring total protease activity in a sample may reveal that altered protease activity is associated with higher or lower antimicrobial activity, giving insight into a possible mechanism for that altered antimicrobial activity. To measure total protease activity, an EnzChek protease assay was performed on a few samples. In this preliminary study, protease activity trended toward higher levels in baseline-positive urine than in baseline-negative urine (Figure 6). Due to limited sample numbers and volumes, further analyses with this assay were not performed.
Figure 6. Urinary protease activity trends toward higher levels in baseline-positive urine than in baseline-negative urine. Preliminary results of protease activity as measured by fluorescence in baseline-positive and baseline-negative urine (baseline-negative, n = 4; baseline-positive n = 6; Bars represent mean ± SEM; Analyzed by Student’s t-test, p = 0.0525)
Urinary Microbiota

AMPs, an arm of host innate immunity, are known to influence the microbiota of various body sites. This may also be true of the urinary tract. Once could easily imagine that disruption of the commensal members of the resident urinary bacterial community might lead to pathological disease. To assess the likelihood of this scenario, we first must characterize the urinary microbiota, attempting to identify “abnormal” or high-risk microbiota profiles. Microbiome analysis was performed in collaboration with Dave Nelson, whose research group performed the amplification and sequencing of bacterial DNA, and Qunfeng Dong, whose group provided the bioinformatic analysis of the resulting data.

Sixty-three urine samples were analyzed for bacterial 16S DNA sequences. Of those 63 samples, 46 (73%) contained high-quality evidence of bacterial DNA, while 17 (27%) samples contain no conclusive evidence of bacterial DNA. In all of the samples with evidence of bacterial DNA, we identified 18 phyla, 100 families, 177 genera, and 1942 operational taxonomic units (OTUs). Figure 7 shows a phylogenetic tree of the two hundred most frequently sequenced OTUs. This illustration, along with the fact that 1,942 OTUs were sequenced in total, demonstrates that bacteria are present and common in urine, in contrast with the previous dogma of sterile urine. Also of interest is the prevalent number of species, or OTUs, that are not associated with any previously identified species. This reveals that our previous view of the urinary tract microbial community, informed by culture techniques, was not comprehensive.
Figure 7. Genetic diversity of urinary microbiota. The two hundred most frequent OTUs in all urine samples analyzed. Due to space limitations, a great number of less frequent OTUs are not shown.
To gain a better picture of the urinary tract microbiota, heat maps were created showing the abundance of sequences of the fifteen most abundant bacterial families (Figure 8) and the twenty most abundant genera (Figure 9) within each sample. These heatmaps revealed that some bacteria tend to be present together. For example, at the family level, Corynebacteriaceae and Incertia Sedis XI tend to occur together. At the genus level, Corynebacterium and Anaerococcus tended to be present together, and Peptoniphilus and Dialister appeared together, also.

To determine if the microbiota of patients in each cohort were similar, we performed a cluster analysis at the family and genus taxonomic levels (Figure 8 and 9). Although the number of baseline culture-positive and postl-UTI samples was small (n=1 and n=2, respectively), it appears that, in terms of their microbiota profiles at the family and genus levels, the two postl-UTI samples were more similar to one another than to any other samples analyzed. At the family level, the two postl-UTI samples also clustered near the one baseline-positive sample. These results indicate that, in a broad sense, their microbiota are similar to the baseline-positive sample. One prominent family that was prevalent in baseline-negative, postl-UTI negative urine and absent in the samples similar to the baseline-positive or postl-UTI positive urine was Lactobacillaceae. Corynebacteriaceae was another family that tended to be present in the urine of baseline-negative patients.
Figure 8. Microbiota profiles of post-UTI and baseline-positive samples at the family level are similar. Hierarchical heatmap showing abundance of bacteria of the fifteen most abundant families. Sequences in these families account for greater than 90% of all sequences in all samples analyzed. Green indicates lower abundance; red indicates higher abundance. Cluster analysis (shown at top of figure) clusters together samples based on similarities in the microbiota profiles using Spearman’s Rank coefficient as a measure of distance.
Figure 9. Microbiota profiles of post-UTI positive samples at the genus level are similar. Hierarchical heatmap showing abundance of the twenty most abundant genera of bacteria. Cluster analysis (shown at top of figure) clusters together samples based on similarities in the microbiota profiles using Spearman’s Rank coefficient as a measure of distance.
Integrated AMP and Microbiota Results

Given that urinary tract infections are likely the result of an imbalance amongst factors of the host immune system, pathogens and commensal bacteria that shape the environment of the urinary tract, studying these different aspects in the context of one another will give us a better understanding of who is at risk for infection and potentially will lead to new therapeutic options and/or prevention strategies.

Because psoriasin is known to exert its antimicrobial effects against *E. coli*, I was interested in whether levels of psoriasin correlated with the presence of *E. coli*. Indeed, psoriasin levels were significantly lower in samples with *E. coli*, detectable either by culture or by 16S sequencing, than in samples with other types of culture-verified infections (Figure 9). However, in the group of “other infections” there appears to be two separate populations, one exhibiting high levels and one exhibiting minimal levels, indicating that there may be other factors at play that we do not currently understand.

Further analysis revealed additional microbiota characteristics that correlate with levels of psoriasin. With the help of our collaborator, Qunfeng Dong, a series of univariate linear regressions were performed examining possible correlations between levels of psoriasin and individual genera of bacteria. By these analyses, we determined that numbers of sequences in two genera, *Streptococcus* and *Peptoniphilus*, each individually correlated with increased levels of psoriasin (Figure 10). Since two genera individually correlated with levels of psoriasin, we next performed a multivariate linear regression analysis to determine if multiple genera together correlated with levels of
psoriasin. A group of seven genera, *Streptococcus, Peptoniphilus, Finegoldia, Varibaculum, Peptostreptococcus, Agromonas* and *Gp3*, was identified whose abundance positively correlated with amounts of psoriasin. It was determined that variation in the abundance of these seven genera accounted for 91% (adjusted R-squared = 0.9069) of the variance in levels of psoriasin (p < 0.0001).

![Figure 10](image)

**Figure 10.** Levels of psoriasin are lower in urine with detectable *E. coli* that in urine with other types of infections. Levels of psoriasin are shown for urine samples with *E. coli* detected either by culture or by 16S sequencing and for urine samples with another type of culture-detected pathogen. Organisms in the “other infection” column include *Staphylococcus aureus, Pseudomonas aeruginosa, Corynebacterium* spp. and *Enterococcus* spp. (Analyzed by Student’s t-test, p < 0.05)
Figure 11. The number of sequences in the genera *Streptococcus* and *Peptoniphilus* each positively correlate with levels of psoriasin. Levels of psoriasin were compared to the number of sequences of either *Streptococcus* or *Peptoniphilus* with a univariate linear regression analysis. *Streptococcus*: $R = .206$, $p = 0.03$; *Peptoniphilus*: $R = 0.182$, $p = 0.042$. 
CHAPTER FOUR
DISCUSSION

Taken together, our data provide some insight into possible interactions between the host, its commensal microbiota and pathogenic microbes. Understanding these interactions may reveal a profile of risk factors that can identify patients who are susceptible to post-UTI. Once we learn the factors that can identify patients at risk, we can begin to develop an assay to assess these measures in patients, and based on those measurements, design a course of treatment meant to specifically address the areas in which the patient is deficient. This assay would serve as an indispensable tool for clinicians.

Sample Processing

Because dilution of the urine can vary greatly depending on patient consumption, concentrations of peptides of interest must be normalized. This normalization permits comparison of values between samples and between cohorts. We decided to normalize AMP levels to total protein. However, our data demonstrated that not all protein assays are appropriate for use with urine, as certain compounds in urine interfere and thus lead to false positive results. For example, some protein assays rely on a mechanism that involves the chelation of metal ions by protein; thus, any other compounds that cause chelation of metal ions, such as uric acid, can lead to a false
positive result. When we compared three commonly used protein assays, a Bradford assay, a modified Lowry assay and a BCA assay, we determined that the Bradford method was the most appropriate and most accurate, whereas the modified Lowry and BCA assays yielded results that were much too high. We henceforth used the Bradford assay to measure protein levels. This finding will be important for all future analyses of any components of the urine.

The discrepancies between the results of these three different protein assays help to explain some other results that I obtained during my attempt to determine the best way to process urine samples for analysis (data not shown). Initially, because I was worried that debris or extra substances in the samples would interfere with many of our experiments, I dialyzed each sample with membranes with approximately 3.5 kD pore sizes. At the time, I was measuring total protein with a BCA assay, and when I measured protein levels in dialyzed versus undialyzed urine, it appeared that dialysis caused a significant loss of protein (e.g. 5 mg/mL down to 100 µg/mL). Knowing what we know now, however, it seems more likely that dialysis actually removed whatever components in the samples were resulting in false positive results with the BCA assay. At the time, though, we decided dialysis was an inappropriate processing step, because it caused too much loss of protein. Now that we know that the BCA assay is an inappropriate method to measure urinary protein, it may be worthwhile to revisit the use of dialysis as a processing step to remove interfering substances. If this method is
used in the future, it may be useful to look into dialysis inserts for 96-well plates that allow for dialysis of many samples at one time.

To separate AMPs from potentially interfering substances, I next experimented with the use of centrifuge filters with pore sizes of 3 kD and 30 kD to separate urine components into three fractions of <3 kD, 3-30 kD and >30 kD. The AMPs would theoretically fall into the 3-30 kD fraction. Using a BCA assay to measure protein, we once again found that most of the protein fell into the <3 kD fraction, although we now think that we were detecting non-protein components.

I also utilized these centrifuge filters to assess whether AMPs in the samples were fully solubilized. To solubilize the peptides, I began to add trifluoroacetic acid (TFA) to samples. I measured hBD-1 concentrations in each fraction of urine samples with and without added TFA. I found that without TFA, most hBD-1 could be found in the >30 kD fraction. However, with the addition of TFA, most of the hBD-1 would be observed in the 3-30 kD fraction (data not shown). This indicated to us that the addition of TFA does help solubilize our peptides of interest, allowing for more accurate measurement by ELISA. However, not all of the hBD-1 could be found in one fraction, even with the addition of acid. To accurately measure AMP concentrations in urine, we decided to forgo the use of centrifuge filters, and simply use a 0.2 µm filter to remove cells and large debris, and then add TFA to solubilize peptides.
Urinary Antimicrobial Peptides

Analysis by ELISA revealed a wide range of hBD-1 levels in the patients who were culture-negative on the day of surgery and did not contract a UTI (baseline-negative), the patients who were culture-positive on the day of surgery (baseline-positive), and the patients who were culture-negative on the day of surgery but became culture-positive after surgery (postI-UTI). Since the distributions were not significantly different, we concluded that hBD-1 could not distinguish the three cohorts.

Likewise, ELISA revealed a wide range of urinary psoriasin in the baseline negative and baseline-positive cohorts. The distribution of the baseline-positive cohort appeared to be bimodal, with some patients expressing relatively high levels of psoriasin and some expressing almost none. In contrast, the level of urinary psoriasin in all postI-UTI patients was minimal. Since psoriasin expression is generally inducible in the presence of infection, it is possible that some patients contracted a UTI (postI-UTI) because they were unable to mount a sufficient response. Unfortunately, low psoriasin concentration could not be used as a predictor of postI-UTI because it was not restricted to the postI-UTI cohort.

Future studies of urinary AMPs should include more samples in the baseline-positive cohort and in the postI-UTI cohorts, as this may reveal distinguishing characteristics in those patients who ultimately come down with infection. It also may be useful to measure levels of cathelicidin, lactoferrin or hepcidin, three peptides with demonstrated antimicrobial activity that have been detected in the urinary tract and
that may play a role during infection. I performed one ELISA for cathelicidin in urine samples. I was not able to detect any measurable amounts (data not shown); however, the ELISA kit had not been validated for use with urine samples. Validating a cathelicidin ELISA for use with urine samples, or determining some other way to quantify urinary cathelicidin, may be useful for future studies.

Running known antimicrobial peptides on a C18 column during HPLC taught us where to expect elution of urinary AMPs. Amphipathic peptides in the urine eluted between 10% and 55% acetonitrile concentration, so we fractionated peptides by collecting fractions at one-minute intervals in that concentration range. This knowledge also will be essential for future urine AMP analyses of this kind. We also observed that baseline-positive urines generally exhibit larger and more frequent peaks in their HPLC tracings, indicating that those samples contain a greater variety and higher concentration of peptides and proteins. This is not unexpected, because during infection the host up-regulates the expression of a variety of genes [12, 14, 15, 23], so one would expect to see a greater number of gene products.

Included in this host of up-regulated gene products are a variety of AMPs and, as expected, the overall antimicrobial activities of baseline-positive urinary peptide fractions against $\Delta mprF S. aureus$ was higher than activities seen in baseline-negative urine. In our small sample size, we did not detect a difference between culture-negative urine from patients who DO NOT contract a postl-UTI (i.e., baseline-negative) and culture-negative urine from patients who DO contract a postl-UTI (i.e. postl-UTI).
Therefore, patients in the postl-UTI cohort could not be identified at baseline based on differences in the antimicrobial activities of their urinary AMPs against ΔmprF S. aureus. These data exclude the idea that the reason these patients contract infection is due to lower antimicrobial activity at the time of surgery; however, these data do not exclude the possibility that patients who develop postl-UTIs may be defective in their ability to mount an appropriate AMP response in response to an infective insult.

With this idea in mind, it is worth mentioning that we only tested the antimicrobial activities of urinary AMPs against the Gram-positive ΔmprF S. aureus. In the future, antimicrobial activities against other relevant bacteria, such as the Gram-negative E. coli, should also be tested. Such a screen would likely reveal differential antimicrobial activity profiles. For example, the urine of baseline-positive patient #817 cultured E. coli but exhibited widely distributed antimicrobial activity against ΔmprF S. aureus. In contrast, the urine of baseline-positive patient #690 also cultured positive for E. coli, but exhibited a very limited range of antimicrobial activity against ΔmprF S. aureus. Another immediate area of future interest would be to identify the components of each fraction by mass spectrometry. This may reveal that certain AMPs are present, but not active, which could explain an individual’s susceptibility to infection. Mass spectrometry also may reveal previously unknown AMPs in the urine.

Because proteases play an integral role in processing AMPs from their pro-peptide forms to their mature forms and in degrading them beyond that, protease activity can be intimately associated with antimicrobial activity. I was able to perform
one preliminary experiment assessing urinary protease activity and found that protease activity trends toward higher levels in baseline-positive urine than in baseline-negative urine. This may indicate that, in addition to up-regulating AMPs, the host also activates or up-regulates proteases to facilitate more processing of AMPs to their active forms. Future studies could expand upon this preliminary experiment to determine if this phenomenon holds true with a larger sample size, and perhaps to see if lower protease activity might identify patients at risk for post-I-UTI before they undergo surgery. To determine the role of certain types of proteases, such as the serine proteases that are responsible for cleaving cathelicidin, a future experiment could include adding inhibitors for these certain classes of proteases, and observing how much of the total protease activity disappears. Any future studies on urinary protease activity also may reveal that it is useful to normalize these results to total protein to account for dilution of the urine due to differences in patient solid and liquid consumption.

**Urinary Microbiota**

In parallel with our initial characterization of the AMP profile of the urinary tract, we have begun to characterize the microbiota profile of the urinary tract. The “normal” urinary tract is generally considered to be a sterile environment. This dogma is based upon culture-based analyses. Using culture-independent DNA-sequencing-based techniques, however, we have shown evidence of bacterial DNA in 73% of the urine samples analyzed. These results are similar to those previously reported by our research team [43-45] and others [46].
A phylogenetic tree of the two hundred most frequent OTUs sequenced provides some indication of the microbial diversity that exists in the lower urinary tract. While many of the OTUs correspond to well-known bacterial genera, many of the most frequent OTUs are unclassified. The fact that so many organisms have not been previously characterized highlights the insufficiency of the culture-based methods upon which researchers, clinical microbiologists and clinicians have relied to identify bacterial residents of the urinary tract [43-46]. This reliance has likely given us a stilted understanding of the microbial profiles of the lower urinary tract, especially in relation to health and disease.

Given the existence of urinary bacterial communities, there likely exist countless interactions among members of this microbiota as well as between the microbes and their hosts. Of these interactions, we know almost nothing. However, given the existence of a urinary microbiota, it might be wise to revisit certain clinical situations. For example, patients with bacterial vaginosis are at higher risk for urinary tract infection [30]. Could it be that the disruption in the vaginal microbiota associated with bacterial vaginosis is of secondary importance compared to a disruption in the urinary microbiota? This idea is supported by the observation that Lactobacillus was often found in the urine of baseline-negative patients who did not contract a postI-UTI, but not in the urine of patients that were baseline-positive or contracted a postI-UTI. If this hypothesis is valid, then antibiotic treatment for bacterial vaginosis could alter the composition of the urinary microbiota, causing the unintended side effect of UTI.
To determine whether the urinary microbiota of patients in a given cohort resembled the microbiota of other patients in that cohort, we performed a cluster analysis and constructed a heat map. At both the family and genus level, patients in the baseline-negative cohort clustered into two general groups. At the family level, these groups appeared to cluster, in part, on the basis of Lactobacillaceae. In contrast, at the genus level, Lactobacillus was not a defining feature. This difference warrants further investigation.

Unfortunately, the number of samples that produced quality sequences for taxonomic classification limited our analysis of the postI-UTI and baseline-positive cohorts. In the end, we only had microbiota profiles for two postI-UTI samples and one baseline-positive sample. It is perhaps telling that, in spite of the small sample size, that the two postI-UTI samples clustered together at both the family and genus levels, indicating that their microbiota profiles are more similar to one another than they are to any other samples. This encouraging result indicates that there may be a characteristic microbiota profile that can identify patients at risk for postI-UTI before they undergo surgery. In addition, at the family level these two postI-UTI samples were relatively similar to the one baseline-positive sample. This similarity may indicate that the postI-UTI patients were already on their way to becoming infected: perhaps they were already colonized with a pathogen, or their urinary microbiota profiles were more conducive for allowing a pathogen to take hold. For example, members of the Lactobacillaceae family were prevalent in many baseline-negative patients who did not develop a postI-UTI, but
absent in samples similar to the post-UTI-positive and culture-positive samples. Perhaps the presence of Lactobacillaceae is indicative of a healthy state. Being able to identify patients that are susceptible to infection would be an important step in developing clinical interventions to prevent these infections from occurring.

We also can interpret the microbiota data by asking which bacteria tend to be present together. At the family level, for example, Corynebacterium and Incertia Sedis XI tend to be present together. At the genus level, Corynebacterium and Anaerococcus tend to pair, and Peptoniphilus and Dialister tend to co-exist. At this time, we are unable to say if these pairings are clinically relevant, but recognizing trends within the microbiota will likely be important in understanding microbiota profiles that are indicative of risk for infection. Further statistical analyses should be performed to determine if there are any groups of bacteria that exhibit significant correlations, either positive or negative, with other groups of bacteria.

Future studies of microbiota profiles should analyze more samples from the post-UTI and baseline-positive cohorts. In addition, it would be useful to analyze the microbiota at a lower taxonomic level, such as the species or strain level. It is entirely possible and likely that certain members of each family and genus are more indicative of risk for post-UTI than the entire family or genus. Indeed, some strains of the species *E. coli* are pathogenic (UPEC) whereas other strains are commensal (e.g. K-12). While 16S rRNA gene sequencing can distinguish genus and in certain cases species, it does not
provide enough depth of information to distinguish between different strains; thus, other molecular techniques will be needed if such a level of depth is required.

**Integrated Analyses**

Analysis of AMPs alone did not reveal any characteristics indicative of risk for postI-UTI. While analysis of microbiota profiles alone revealed some interesting microbiota profiles that could potentially identify patients at risk for postI-UTI, this line of investigation requires further exploration. We therefore sought to integrate what we know about both factors in the urinary tract. The goal was to reveal relationships that might identify patients at-risk for postI-UTI.

Because psoriasin has been shown to exert its antimicrobial effects against *E. coli*, and because *E. coli* is such a prevalent uropathogen, appearing several times in our culture-positive samples, we wondered whether levels of psoriasin corresponded with samples containing *E. coli*, detectable either by culture techniques or by 16S rRNA gene sequencing. Psoriasin levels were significantly lower in samples containing *E. coli* than in other culture-positive samples. This observation is consistent with the notion that patients contract an *E. coli* infection because they are not able to express a sufficient antimicrobial response to the initial *E. coli* insult. Indeed, similar results were found in the lung, where psoriasin levels were found to be elevated in the presence of *S. aureus*, but not elevated in the presence of *E. coli* as detected by culturing bronchoalveolar lavage fluid [85].
Our collaborator Qunfeng Dong performed univariate linear regression analysis on the numbers of sequences of any single genus and the detected levels of psoriasin. In doing so, he discovered that the numbers of sequences of two genera, *Streptococcus* and *Peptoniphilus*, each positively correlated with levels of psoriasin. A multivariate linear regression analysis, which is able to account for the combined effect of multiple genera on the levels of psorasin, revealed that the numbers of sequences of any of a group of seven genera (*Streptococcus, Peptoniphilus, Finegoldia, Varibaculum, Peptostreptococcus, Agromonas* and *Gp3*) positively correlated with the levels of psorasin. In fact, variance in the number of sequences of these genera could account for approximately 91% of the variation in psoriasin concentration. Psoriasin’s positive correlation with this defined group of bacteria, and its conspicuous absence in any samples containing *E. coli* could indicate that a microbiota containing a combination of bacteria of these seven genera is protective against *E. coli* infection. It is possible that they provide protection by inducing the expression of psoriasin, which is prohibitive for the colonization and proliferation of *E. coli*. Such an interaction would mirror interactions at other body sites, where commensal bacteria induce the expression of AMPs, consequently protecting the host from infection and removing potential competitors from the environment, such as *Staphylococcus epidermidis* does on the skin [79].

There are any number of future directions in the area of integrated analysis of AMPs and the microbiota, not the least of which is performing further statistical
analyses on the data we already have. There are likely other correlations that could shed light on important interactions; we just need to continue to thoughtfully analyze the data to glean from it what we can. There are a few other specific questions to address in the future. One is whether certain genera of bacteria correlate with any other AMPs besides psoriasin. We could also ask more mechanistic questions about how these genera induce psoriasin or any other AMP. Other future experiments could involve using either an animal model of UTI or an *in vitro* system to ask whether the presence of any genera associated with higher AMPs is protective against *E. coli* infection or any other type of infection.

If we can identify a profile of urinary AMPs and/or microbiota that is associated with higher risk for postI-UTI, it would then become crucial to develop a tool for clinicians to rapidly assess levels of AMPs and certain microbes in a single assay. One possibility would be the development of a multiplex assay that utilizes fluorescent quantum dots to quantitatively measure levels of different AMPs and levels of bacterial DNA from certain different bacteria all in the same assay. If there is more than one profile associated with risk, or if there are different profiles associated with risk for different types of infection, we could develop a course of treatment that specifically aims to correct the area in which that patient is deficient.

Importantly, it may prove beneficial to shift our focus away from eradication as a means of treating infection, and instead treat each body site like the ecologically distinct environment that it is, and rather focus on maintaining a healthy ecology. In support of
this idea is the successful treatment of pathogen-associated antibiotic-associated diarrhea with donor stool samples [39]. This provides an example of how microflora disrupted by antibiotics providing an ecological niche for a pathogen to take hold can be restored to a healthy state by reintroducing a healthy balance of microorganisms rather than by simply trying to eradicate the pathogen. We may find that a good way to treat and/or prevent urinary tract infections in POP/UI surgery patients is to optimize the urinary microbiota, perhaps by introducing probiotics to establish populations of bacteria associated with a healthy state for the host.

Approaching it from a different angle, we may find that providing exogenous AMPs or artificially inducing expression of AMPs is a more effective way of preventing infection than prophylactic antibiotics. Another interesting dynamic of AMPs and host defense is the report that different people have different copy numbers of hBD-2 encoded in their genomes. Low hBD-2 copy number has been linked to Crohn’s disease, psoriasis and COPD [86], but no similar analysis of hBD-2 copy number has been performed in relationship to susceptibility to UTI. This could potentially be an interesting area of future research.

As our understanding grows of the processes and interactions that lead to UTI, there is a great need to translate our research knowledge into clinical practice. The strikingly high rate of postl-UTI highlights the need for inventive new therapeutics. Any areas of future research that seek to translate our understanding into tools that can be utilized by clinicians will yield great benefits for patients.
REFERENCE LIST


VITA

Vanessa Nienhouse was born on August 14, 1988 in Elgin, Illinois to Laurence and Caryn Nienhouse. For her undergraduate education, she attended Augustana College in Rock Island, Illinois, where she earned a Bachelor of Arts in Biology, with a minor in German, *cum laude*, in 2010.

In August of 2010, Vanessa began her graduate studies at Loyola in the program of Infectious Disease and Immunology. She joined Dr. Alan Wolfe’s lab where she studied the urinary microbiota and antimicrobial peptides in the urinary tract. While at Loyola, Vanessa was a member of the Group on the Urinary Microbiome. She also volunteered at community public health events including a public health fair held at First Baptist Church in Elmhurst and a breast cancer educational event held at Casa Puertorriquena in Chicago.