THE IMPACT OF POLYMICROBIAL INFECTIONS ON URINARY TRACT INFECTIONS & ANTIBIOTIC RESISTANCE

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TRADITIONAL CULTURE LIMITATIONS

Urinary tract infections (UTIs) result in ~10.5 million office visits in the US every year with an additional 1 million ER visits which result in approximately 100,000 hospitalizations.\(^1\) Populations at risk include pregnant women, the elderly, patients with underlying urologic conditions, infants, etc. In elderly populations, genitourinary infections are the second most common infection with direct costs (in 1995) of $659 million and indirect costs of ~$936 million with total annual costs of $2 billion.\(^2,3\)

The introduction of molecular technologies, for the detection and characterization of microbes in the urinary tract is the necessary next step in the evolution of better clinical management of urinary tract disease.

The current gold standard for the detection of microbes contributing to UTIs is traditional culture which hasn’t notably changed since it originated as meat infusion broths in 1865.\(^4\) Robert Koch evaluated a number of solid media and settled on meat extract combined with gelatin poured on glass plates.\(^5\) Subsequent research resulted in the incorporation of agar (polysaccharide derived from seaweed) to provide the solid support. Combined with the introduction of updated nutrients and modified glass supports, traditional agar plate media was in use by the 1890s. Although selective media has evolved over time, the general methodology has not significantly changed in a hundred years.

Traditional culture’s reputation as the gold standard began to be challenged when molecular methods polymerase chain reaction (PCR) demonstrated that a wide variety of microbes, not found by culture, exist in the urine from female patients that did not present with the clinical definition of a UTI.\(^6\) Urine, previously thought to be sterile, was now understood to have undetected microbes.\(^7\) In hindsight, this should not have been a complete surprise because culture, by its nature, is biased towards faster-growing microorganisms (such as *Escherichia coli* or *Enterococcus faecalis*) that thrive in aerobic conditions, as opposed to slow growing organisms that are either fastidious or grow under anaerobic conditions (*Lactobacillus*, *Ureaplasma*, etc.).\(^8,9\)

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Enhanced culture methods were subsequently developed with the aim of detecting more and varied organisms; culture modification’s included plating larger volumes of urine, varying atmospheric conditions and longer incubation periods. The enhanced culture approach (EQUC) found thirty-five genera and eight-five species, 92% of which were reported as no growth by the standard urine protocol.\(^10\) In addition, a study by Loyola and the University of Texas expanded the already superior culture approach (EQUC) by additional plating conditions (BAP, chocolate, colistin-nalidixic acid agars and a variety of atmospheric conditions, etc.). They examined the flora from bladders of woman that did/did not meet the clinical UTI definition and discovered that traditional, current culture misses 67% of uropathogens overall and even missed 50% of organisms in patients with severe urinary symptoms.\(^11\)
THE MICROBIOME

Although urine was, until recently, considered a sterile environment, significant research began to establish that microbes exist in communities in a variety of human systems such as the gut. In 2008, the National Institute of Health (NIH) initiated the Human Microbiome Project (HMP). The HMP mission is to provide the resources necessary to allow the wide-ranging characterization of the microbial communities of the human body (nasal passages, oral cavity, skin, gastrointestinal tract, and urogenital tract) and analyze their role in human health and disease—however, the bladder was not initially included in the project perhaps because it was assumed to be sterile or that it was considered unethical to obtain bladder biopsies or suprapubic aspirates from healthy individuals.8,12,13 The investigation of a microbiome in the bladder lagged behind other systems because of the limited ability to culture microorganisms leading to a misunderstanding about the nature, frequency and scope of the bladder microbial community and its relationship to UTIs. However, we now know that the urinary tract has its own unique microbiota with multiple studies categorizing the urinary bacterial community.9,13

Using molecular tools, we now also know that the composition of a bacterial community can have clinical relevance. As an example, research demonstrates a substantial increase in Lactobacillus in interstitial cystitis (painful bladder syndrome). In culture, Lactobacillus requires 48 hours incubation in 7% CO² to be detected which may be why interstitial cystitis samples are not typically associated with bacterial growth.14 In urgency urinary incontinence (UUI), an increase in UUI clinical severity is correlated with a decrease in diversity of the microbiota.15

POLYMICROBIAL INTERACTIONS

Traditionally, microbiological research has focused on mono-microbial infections—one organism at a time. This has largely been due to the relative ease of experimental approaches focused on one microbe ostensibly responsible for one infection. In reality, most infections are polymicrobial in nature—some studies have shown that as many as 39% of urine cultures from elderly patients have polymicrobial infections.16 Understanding that infections are polymicrobial in nature is not new. Since the days of Pasteur, it was hypothesized that some microbes were not easily cultivated but still contributed to infection.17 Polymicrobial infections can be distinguished in three ways: (1) Dysbiosis: changes in the composition of individual species within the bacterial community. (2) Pathogenic colonization (a pathogenic organism colonizes a site already inhabited by commensal microbes. (3) An organism colonizes a site it does not normally inhabit.

Bacterial communities inhabiting human systems, such as the bladder, can be thought of as cities: three dimensional structures in which the location and spatial organization of the organisms plays an important role in virulence.18 Some studies have even shown that mixed infections in biofilms are more robust to antimicrobial agents than individual species encased in a biofilm.19 Other studies have demonstrated that E. coli can invade bladder epithelial cells and create intracellular bacterial reservoirs that can last for substantial periods of time.20 Two or more of the individual species within these three dimensional in situ structures can result in worse disease when compared to either microbe acting alone: this is considered synergy and polymicrobial infections are considered to be worse than mono-microbial infections with experimental evidence in urinary tract infections in model systems as well as clinical isolates.17,21 Croxall et al., compared clinical UTI isolates from patients with either poly- or mono-microbial infections and found that E. coli from mixed cultures were ~1,000x more invasive than the reference strain.22 In UTIs with a primary uropathogenic microbe, other microbes at low levels (<10⁵ CFU/mL of urine) have not been thought to be clinically relevant. However, Kline et al., have demonstrated that this a flawed thought process. In their murine-model research, low titers of group B Streptococcus may supress host immune processes creating a more favorable environment for uropathogenic organisms.23

Indeed, bacteria can not only interact with other bacterial species they can also cooperate with fungi to produce enhanced infections: Candida albicans, a fungal pathogen has been found to
coexist in polymicrobial infections in biofilm structures: these mixed infections are associated with amplified frequency and disease severity.\textsuperscript{24} In the urinary tract, \textit{E. coli} contributes to the ability of \textit{C. albicans} to attach to the bladder lining which the organism was unable to do on its own. \textit{Acinetobacter baumannii}, a hospital associated pathogen, also often interacts with \textit{C. albicans} and is a problem for critically-ill patients on ventilation with urinary catheters.\textsuperscript{25} There are multiple ways discrete species act in synergistic ways to produce enhanced infections. Although, many organisms have distinct and separate metabolic pathways, experimental evidence in urinary infections that co-infection with \textit{E. coli} and \textit{Proteus mirabilis} enhances urinary tract colonization and pathogen persistence.\textsuperscript{26} Additionally, species can cross feed: use metabolites from a different species, such as complex carbohydrates, as a fuel source.\textsuperscript{27} Microbes also, produce low molecular weight signals (known as quorum sensing) that enable them to ‘cross talk’ with other cells of the same bacterial species to coordinate their activities. However, these signals can actually be listened to (eavesdropping) and answered by unrelated species.\textsuperscript{17}

**POLYMICROBIAL INFECTIONS IMPACT ON ANTIBIOTIC RESISTANCE**

Antibiotic resistance is a well-known problem and currently contributes to ~23,000 deaths per year. Polymicrobial infections not only can enhance virulence, they also can effect changes in how infections respond to antibiotic therapy.\textsuperscript{28} Bacteria become transcriptionally active during antibiotic treatment and research shows that antibiotics can impact the composition of the microbiome.\textsuperscript{29} In UTIs, polymicrobial infections can often be considered probable contamination and not properly assessed. Combined with traditional culture’s inability to detect many organisms, an incomplete picture of the patient’s urinary microbial landscape can lead to ineffective therapy.

There are multiple mechanisms by which polymicrobial infections respond to antibiotic treatment- many of which remain to be elucidated. In some studies, three dimensional structures such as biofilms encapsulate bacteria in an extracellular matrix that can potentially provide protection against antibiotics.\textsuperscript{24,30} \textit{Staphylococcus aureus} encased in biofilms with \textit{C. albicans} has been shown to have enhanced resistance to vancomycin.\textsuperscript{31}

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Bacterial communication also plays an important role in mixed communities and can impact response to antibiotic therapy. In otitis media, signaling between bacterial species promotes resistance to antibiotics and persistence.\textsuperscript{32} The intestinal pathogen, \textit{Salmonella typhimurium}, becomes more antibiotic resistant in response to the signaling molecule indole produced by other bacterial species. Although \textit{S. typhimurium} does not produce indole, when exposed to exogenous indole or indole produced by \textit{E. coli} in a mixed community, \textit{S. typhimurium} becomes more able to tolerate antibiotics. Indole signaling as well as other signaling mechanisms may affect the ability of pathogens in polymicrobial infections to resist antibiotics, persist and form chronic infections.\textsuperscript{33} In mixed cultures, \textit{Stenotrophomonas maltophilia} produces a diffusible signal factor that changes the morphology of neighboring \textit{Pseudomonas aeruginosa} which can be associated to a specific gene (rpfF): this signaling factor produced by \textit{S. maltophilia} increases the tolerance of \textit{P. aeruginosa} to polymyxins B and E.\textsuperscript{34} In model systems using 3D printing technology,
“Species act in synergistic ways to produce enhanced infections.”

In urinary tract infections, polymicrobial in elderly patients typically consists of up to five organisms. De Vos et al., examined the interactions between 72 bacterial isolates from elderly people with UTI symptoms. They measured bacterial growth via optical density in an artificial urine medium and quantified the interactions between the species. They found that most interactions resulted in no change in growth but 18% of all species to species interactions enhanced growth and 40% of these positive interactions led to >2x growth. Conversely, 23% of interactions resulted in negative growth. They also measured the impact of species to species interactions on antibiotic efficacy. They assessed organism’s growth in response to two commonly used antibiotics for UTIs (trimethoprim-sulfamethoxazole and nitrofurantoin). Using media conditioned by donor isolates, they observed that clinical isolates often protected each other from the antibiotics: 25% of tested species to species interactions demonstrated greater than a 3.5 fold increase in tolerance for trimethoprim-sulfamethoxazole but decreases of the same magnitude only occurred in 12% of results. Similar results were observed for nitrofurantoin. They also noted that clinical isolates from the same community tended to protect each other slightly more than isolates from different communities.
**GUIDANCE MOLECULAR TESTING**

GUIDANCE combines two critically important assessments to provide a nuanced view of the polymicrobial nature of UTIs and their potential response to a variety of possible antibiotic therapies. GUIDANCE uses polymerase chain reaction (PCR) to detect DNA from twenty-five (seven) pathogens for UTIs and twenty-seven pathogens for prostatitis (Table 1) and can detect organisms as low as 1,620 – 5,401 cells per milliliter (depending on the organism) to as high as >6 million cells/mL.

In an internal analysis, GUIDANCE was compared head-to-head to traditional urine culture (gold standard) for the ability to detect organisms causing UTIs. (All organisms detected by either method were at a threshold of 10,000 CFU of cells/ml). As seen in Figure 1, the GUIDANCE PCR-based assay had significant improvements in accuracy over culture in correctly finding pathogens in urine samples from UTIs: sensitivity (97% vs 31%), over culture with only 3% misdiagnosed compared to 69% for urine culture.

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**Table 1. GUIDANCE Pathogen Detection for UTIs**

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<thead>
<tr>
<th>Pathogen</th>
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<th>Pathogen</th>
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<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td>Corynebacterium riegelii</td>
<td>Proteus mirabilis</td>
</tr>
<tr>
<td>Actinobaculum schaalii</td>
<td>Corynebacterium urealyticum</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>Aerococcus urinae</td>
<td>Enterobacter aerogenes</td>
<td>Serratia marcescens</td>
</tr>
<tr>
<td>Alloscardovia omnicolens</td>
<td>Enterococcus faecalis</td>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>Escherichia coli</td>
<td>Staphylococcus saprophyticus</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>Klebsiella oxytoca</td>
<td>Streptococcus agalactiae</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>Streptococcus anginosus</td>
<td></td>
</tr>
<tr>
<td>Citrobacter freundii</td>
<td>Morganella morganii</td>
<td></td>
</tr>
<tr>
<td>Citrobacter koseri</td>
<td>Mycoplasma genitalium</td>
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**Misdiagnosed Positive Cases (N=207)**

<table>
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<tr>
<th></th>
<th>Guidance UGx</th>
<th>Urine Culture</th>
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<tr>
<td>Misdiagnosed Positive</td>
<td>3%</td>
<td>69%</td>
</tr>
<tr>
<td>Positive Cases</td>
<td>97%</td>
<td>31%</td>
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**Accuracy (Positive Cases, N=207)**

<table>
<thead>
<tr>
<th></th>
<th>Guidance UGx</th>
<th>Urine Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>97%</td>
<td>31%</td>
</tr>
</tbody>
</table>
Table 2.
- Ampicillin
- Ampicillin/Sulbactam
- Augmentin
- Cefazolin
- Cefepime
- Cefoxitin
- Ceftazidime
- Ceftriaxone
- Cefaclor
- Ciprofloxacin
- Gentamicin
- Levofloxacin
- Nitrofurantoin
- Piperacillin/Tazobactam
- Tetracycline
- Trimethoprim/Sulfamethoxazole
- Vancomycin

**ANTIBIOTIC RESISTANCE (ABR) ASSAY**

This assay measures optical density with a spectrophotometer setting a threshold value to measure growth of organisms in the ‘soup’, or polymicrobial mixture from the actual patient. The benefit of the ‘soup’ approach is that it allows real-world antibiotic sensitivity assessment of the polymicrobial community from the patient’s UTI. Interaction between species that may result in unexpected antibiotic resistance may more likely be detected via this phenotypic assessment. The minimal inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test. Optical density measurements greater than or equal to the threshold are designated Resistant (R) meaning bacterial organisms present in a patient sample were resistant to that particular antibiotic at that concentration. Measurements less than this threshold are designated Sensitive (S) meaning bacterial organisms present in a patient sample were sensitive to that particular antibiotic at that concentration. The GUIDANCE assay currently tests for eighteen antibiotics (Table 2) and the list continues to grow.

**POLYMICROBIAL INFECTIONS ASSESSED WITH MOLECULAR TOOLS**

As mentioned previously, some studies have shown that 39% of urine cultures from elderly patients have polymicrobial infections. But our analysis of over two thousand cases suggests that polymicrobial infections occur much more frequently (Figure 2) with as many of 68% of cases having infections with more than one microbe. Indeed, 27% of these cases had at least four organisms. This is interesting in the context of the published data from De Vos et al, in which 18% of species to species interactions enhanced growth with 40% of these positive interactions leading to at least twice the growth as well as the fact that 25% of tested species to species interactions demonstrated greater than a 3.5-fold increase in tolerance for trimethoprim-sulfamethoxazole.36

![Figure 2. Summary of GUIDANCE urine testing for prostatitis (PRx) and urinary tract infection (GUIDANCE UGx) results. A total of 2,246 cases with infection were evaluated. Of these, 32% (720 of 2,246) were positive for a single pathogen while 23%, 18% and 27% of the cases were positive for 2, 3, or 4 or more pathogens.](image-url)
A closer examination of laboratory co-infections (internal data) demonstrates that there is wide variability in both the percentage and composition of polymicrobial coinfections. *E. coli*, typically the most abundant organism in urinary tract infections had relatively equal distribution of coinfections from singular infections to as many as five organisms with the majority (78%) being polymicrobial. In some cases, exemplified by *Corynebacterium riegelli*, *Alloscardovia omnicolens*, *Proteus mirabilis*, *Morganella morganii* and *Klebsiella pneumoniae* - a significant percentage of infections had at least five organisms – 65%, 46%, 41%, 50% and 33% respectively. When reviewing fungal infections, the same results can be observed. As an example, 82% of *Candida albicans* infections were polymicrobial with 23% having five or more organisms (Figure 3).

In some cases (internal data), there is preliminary evidence of paired organisms having enhanced resistance to specific antibiotics compared to antibiotic resistance observed with either organism alone (Figure 4a and 4b).

In Figure 4a, a mixture of *E. coli* and *Klebsiella pneumoniae* from patients with symptomatic urinary tract infection were associated with enhanced resistance to Ampicillin/Sulbactam and Piperacillin/Tazobactam combinations. In Figure 4b, a mixture of *E. coli* and *Enterococcus faecalis* from patients with symptomatic urinary tract infection were associated with enhanced resistance to Ampicillin or Nitrofurantoin.

These results highlight how polymicrobial infections may negatively impact on clinical management and the importance of using the proper assay. Mutualistic infections resulting in increased antibiotic resistance would not be captured by culture or simple DNA detection which assesses the presence or absence of resistance genes: only a molecular assay, like GUIDANCE which does both - combines DNA detection with phenotypic characterization - would provide the most complete information to the clinician.
**ANTIBIOTIC RESISTANCE (ABR) ASSAY**

In the United States, UTIs result in millions of office and ER visits with annual costs in excess of $1 billion. Although culture, the current laboratory gold standard for microbe detection, hasn’t significantly changed since the mid-nineteenth century, our scientific knowledge and perspective on urine as a sterile environment has evolved. Urine is no longer considered sterile and now is thought to have a microbiome in which infections are polymicrobial and mutualistic in nature: this has implications for antibiotic resistance. Current culture misses ~67% of uropathogens and new approaches are required. The GUIDANCE molecular assay has demonstrably higher pathogen detection than culture and combined with its unique phenotypic assay component, GUIDANCE allows for more informed patient management.
ABOUT PATHNOSTICS

Pathnostics is a diagnostic solutions company that pioneers approaches for better patient care. Our nimble team of experts develop solutions to address diagnostic and therapeutic dilemmas because we care about a better future for physicians, patients, and humanity.

We lead the way to redefining what is possible in patient care by identifying problems that others don’t see. By providing expert treatment options, including an emphasis on antibiotic stewardship, we empower physicians to champion patient health today and in the future.

POLYMICROBIAL INFECTIONS IMPACT ON ANTIBIOTIC RESISTANCE