Utility of DNA Next-Generation Sequencing and Expanded Quantitative Urine Culture in Diagnosis and Management of Chronic or Persistent Lower Urinary Tract Symptoms

Monika Gasiorrek,^a Michael H. Hsieh,^b Catherine S. Forster^c

^aUniversity of Texas Health Science Center, San Antonio, Texas, USA

^bDivision of Urology, Children's National Health System, Washington, DC, USA

^cDepartment of Pediatrics, Children's National Health System, Washington, DC, USA

ABSTRACT Many patients suffer from chronic, irritative lower urinary tract symptoms (LUTS). The evaluation and management of these patients have proven difficult with the use of standard diagnostic tools, including urinalysis and urine culture. The growing body of literature on the urinary microbiome has looked at the possible implications of the bladder microbiome and dysbiosis, or perturbations in the microbiome, in conditions associated with chronic LUTS. Disorders such as recurrent urinary tract infections (UTIs) and interstitial cystitis have been studied utilizing 16S rRNA rapid next-generation gene sequencing (NGS) and expanded quantitative urine culture (EQUC). In this article, we first present a brief review of the literature describing the current understanding of the urinary microbiome and the features and applications of NGS and EQUC. Next, we discuss the conditions most commonly associated with chronic, persistent LUTS and present the limitations of current diagnostic practices utilized in this patient population. We then review the limited data available surrounding treatment efficacy and clinical outcomes in patients who have been managed based on results provided by these two recently established diagnostic tools (DNA NGS and/or EQUC). Finally, we propose a variety of clinical scenarios in which the use of these two techniques may affect patients' clinical outcomes.

KEYWORDS DNA sequencing, urinary microbiome, urinary tract infection

Many patients suffer from chronic, irritative lower urinary tract symptoms (LUTS), such as urgency, frequency, nocturia, urge incontinence, urethral pain, and bladder pressure and pain. Chronic, irritative LUTS are often attributed to diagnoses such as recurrent urinary tract infections (UTIs) or the less understood phenomenon of interstitial cystitis/painful bladder syndrome (IC/PBS). The standard evaluation of LUTS, which typically involves urinalysis and reflex urine culture, has restricted utility in these patients. This is due to the limitations of these diagnostic tools in the context of evolving knowledge about the role of the urinary microbiome, or the collection of resident microbial genomes within the urinary bladder, in urologic disease. Furthermore, management of these conditions has sparked controversy in the age of antibiotic stewardship. There is a constant question of whether frequent antibiotic use does more harm than good, particularly in this patient population (1).

The urinary microbiome presents a unique opportunity to improve our understanding of these conditions, and potentially optimize treatment. The use of 16S rRNA rapid next-generation gene sequencing (NGS) has demonstrated that the bladder is not a sterile environment, but rather home to its own unique microbiome (2–7). Expanded quantitative urine culture (EQUC) was subsequently developed to cultivate these microorganisms (3). EQUC has been shown to detect up to 92% of species not

Citation Gasiorrek M, Hsieh MH, Forster CS. 2020. Utility of DNA next-generation sequencing and expanded quantitative urine culture in diagnosis and management of chronic or persistent lower urinary tract symptoms. *J Clin Microbiol* 58:e00204-19. <https://doi.org/10.1128/JCM.00204-19>.

Editor Colleen Suzanne Kraft, Emory University

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Catherine S. Forster, csforster@childrensnational.org.

Accepted manuscript posted online 16 October 2019

Published 23 December 2019

TABLE 1 DNA NGS and EQUC test characteristics and supporting evidence^a

Test	Description	Evidence	Limitations
NGS	<p>PCR amplification and Next Generation Sequencing (NGS), or high-throughput sequencing, of essential 16S ribosomal RNA genes.</p> <p>Takes advantage of the nine known hypervariable regions of the otherwise highly conserved 16S rRNA amplicon to distinguish even closely related bacterial species through evolutionary polymorphisms.</p>	Pearce et al [5]: Bacterial DNA was detected via 16S rRNA gene sequencing in 64.9% (48/74) of collected urine samples, while standard urine culture had a false negative rate of around 90%	Universal PCR primers limit the read lengths of the hypervariable regions, making it difficult to always distinguish between species.
		McDonald et al [10]: 100% of symptomatic patients had DNA sequencing results positive for urinary bacteria (n=44), while only 30% has positive results on standard urine culture. Symptom score improvement was significantly higher in patients treated based on NGS results (n=22), compared to patients treated based on standard culture findings (n=22).	Different PCR primer pairs target different regions of the 16S rRNA gene sequences, which can result in variable microbiome profiles for the same sample.
		Pearce et al [20]: 51.1% of urine samples from patients with UII (n=182) and no signs of infection (negative nitrite and leukocyte esterase on dipstick) demonstrated bacterial DNA by NGS.	Variation in quality and composition of the procedures and resources used for data analysis following sequencing and amplification, such as the 16S rRNA databases utilized, resulting in significant differences in microbiome analyses.
EQUC	<p>Plating a larger urine sample on a variety of media at different growth temperatures and under both aerobic and anaerobic conditions, incubated for extended periods of times.</p> <p>Involves plating 100 μL of urine on MacConkey, blood, as well as colistin-nalidixic acid agars, and placement in a 5% CO₂ incubator at 35°C for 48 hours. This compares to the standard culture technique utilized by most labs, where 1 μL of urine is plated on MacConkey and 5% sheep blood agar plates under standard ambient atmospheric, aerobic conditions at 35°C for 24 hrs.</p>	Hilt et al [3]: 80% of urine samples from 65 patients (n=41 OAB, n=24 controls) grew bacteria using the EQUC protocol. 92% of these EQUC-positive samples demonstrated no growth on standard urine culture. The concordance with DNA NGS is as high as 80%.	Antibiotic resistance gene data obtained through NGS can only used to infer or predict phenotypic antibiotic resistance patterns. Confirmatory standardized growth-based susceptibility testing may still be necessary.
		Pearce et al [5]: 78.9% of urine samples assessed via EQUC grew bacteria. 90.1% of these EQUC-positive samples demonstrated no growth by standard urine culture.	Availability and accessibility.
		Price et al [8]: Standard urine culture detected only 33% of bacteria found using an expanded-spectrum EQUC protocol. 41% of patients treated for 3-7 days based on standard urine culture results reported same or worse symptoms. 50% of these patients had 1 or more uropathogens missed by standard urine culture.	Time and resource intensive, as compared to standard cultures
			Availability and accessibility

^aSee references 3, 5, 8, 10, and 20.

otherwise grown by standard urine culture methods in both asymptomatic and symptomatic patients with LUTS (3, 5, 8). Other groups have also developed alternative enhanced culture methods, similar but not identical to the standard EQUC (4). Many studies have used either NGS or EQUC to look at the possible associations of the bladder microbiome and dysbiosis, defined by Brubaker and Wolfe as “an unhealthy perturbation in the normal bacterial community of a particular niche (e.g., the bladder),” in conditions associated with chronic LUTS (9). However, there are very few studies investigating clinical outcomes of patients whose clinical symptoms have been managed based on the results of either DNA NGS and/or EQUC (4, 6, 10, 11). In this article, we present a review of the current literature describing the benefits of DNA NGS and EQUC in clinical scenarios where the utility of standard diagnostic procedures may be limited (Table 1).

Bladder/urinary microbiome. In 2008, the National Institutes of Health’s Human Microbiome Project characterized the resident microbiome in various sites of the body, including the gastrointestinal (GI) tract, vagina, skin, and nasal cavity. The purpose of

this project was to better understand the role of endogenous microorganisms in health and disease (11). The urinary microbiome, however, was not included in the results of the initial phase of the project (11). With the development of new diagnostic technologies, namely, NGS, it became evident that the concept of urine sterility was limited by both our working definition of a UTI, as well as the standard urine culture used to either confirm or rule out disease. With the development of high-throughput DNA sequencing that takes advantage of bacterial 16S rRNA, many research teams have demonstrated that neither the bladder (2–6, 11) nor urine (12, 13) is sterile, but they are in fact home to an extensive and diverse microbiome regardless of symptom status. It should be noted that the bladder microbiome is sampled through catheterized urine rather than voided urine as catheterized and voided urine samples have distinct microbial niches (2, 14). Questions remain, however, about the clinical utility of analysis of the urinary microbiome. Furthermore, what constitutes a normal urinary microbiome, including exact species composition, range of species, and diversity, as well its changes with age, hormonal status, race, and/or ethnicity, is an emerging area of research (7, 13). Current literature describes the urinary microbiome as having a low biomass, consisting of 10^2 to 10^5 CFU/ml of urine, with anywhere from one to dozens of species comprised primarily of the genera *Lactobacillus*, *Gardnerella*, *Streptococcus*, *Staphylococcus*, and *Corynebacterium* (1). Research into the characterization of the specific role of the urinary microbiome in genitourinary disease states and the application of this knowledge in disease treatment and prevention are also ongoing and discussed in the sections below. Concurrently, there is a need for future studies to assess the utility of NGS and/or EQUC outside of the research lab and in the clinical setting.

DNA next-generation sequencing. The culture-independent DNA-based identification of microorganisms was developed by microbial ecologists in order to detect bacterial species without the need for culture. One technique utilizes PCR amplification and high-throughput sequencing of essential 16S rRNA genes, a form of NGS. More specifically, this technique takes advantage of the nine known hypervariable regions of the otherwise highly conserved 16S rRNA gene amplicon to distinguish even closely related bacterial species through evolutionary polymorphisms (9, 11). There are currently several companies that offer NGS for the purpose of clinical diagnosis of urologic pathology, including MicroGen DX and Aperiomics. Norgen Biotek Corp. is a company based in Ontario, Canada, that provides similar services in partnership with Houston-based Diversigen Inc. The clinical utility of these tests, however, is not established.

In addition to disproving the theory that the bladder is a sterile environment (9), the use of NGS-based technologies demonstrates that standard diagnostic methods, such as urinalysis with reflex urine culture, lack the sensitivity and specificity for proper detection of all urinary organisms. For example, one study that compared standard urine culture with next-generation DNA sequencing found that 100% of symptomatic patients had DNA sequencing results positive for urinary bacteria ($n = 44$), while only 30% had positive results on standard urine culture (10). Other groups have confirmed these findings, with Pearce et al. demonstrating that bacterial DNA was detected via 16S rRNA gene sequencing in 64.9% (48/74) of collected urine samples, while standard urine culture had a false-negative rate of around 90% (3, 5). Notably, the cost of 16S rRNA gene sequencing by MicroGen DX DNA sequencing is approximately 200 dollars. Due to the limitations of this technology, including interpretability and inability to obtain any information on antibiotic resistance genes, it is important to also account for the additional cost of growth-based susceptibility testing following NGS at this time. A standard urine culture can cost around 60 dollars, which increases to approximately 500 dollars with additional testing for fungal or anaerobic species (10). Gaitonde et al. used time-driven activity-based costing to quantify the cost of both evaluating and managing recurrent urinary tract infections (UTIs) in women. They found that the cost of the work up itself ranges from 390 to 730 dollars, depending on the use of imaging. The subsequent cost of acute management with antibiotics ranges from 10 dollars for oral trimethoprim-sulfamethoxazole (TMP-SMX) to as high as approximately 4,000 dollars

for intravenous (i.v.) ertapenam, depending on the patient's drug allergies or resistance status of the causative organism. The cost of self-start, postcoital, or continuous antibiotics for the management of recurrent UTIs ranges from 40 to 190 dollars per year. The study also included the cost of conservative management with interventions with variable efficacy, such as D-mannose, *Lactobacillus*, cranberry tablets, or estrogen therapy, which range from 50 to 1,300 dollars a year (15). Thus, in the appropriate population and with further refinement, 16S rRNA NGS may prove cost-effective as a diagnostic tool.

While promising, there are limitations to the use of these DNA-based techniques to identify the components of the urinary microbiome. The DNA sequencing workflow of a urine sample involves many steps, including microbial DNA isolation and purification, followed by 16S rRNA amplification and sequencing, and finally bioinformatic analysis of the sequencing data using various software database platforms. As a result, this technology continues to have many limitations, especially with regard to its clinical applications. One of the biggest shortcomings of this method is the process of PCR amplification using "universal" PCR primers that anchor the variable regions of the 16S rRNA of most bacteria. This allows for the sequencing and amplification of these hypervariable sequences in order to distinguish bacteria at the phylum, genus, and species levels. However, the sequencing platform used dictates the read length of these primers. This subsequently limits the read lengths of the hypervariable regions that are used for the resolution of one bacterium from another. Just a short section of the hypervariable region is ultimately sequenced, as dictated by the primer. Since many different bacteria may share the same short amplified sequence, some closely related bacterial taxa ultimately cannot be distinguished from one another (16). Nevertheless, Gao et al. have developed a Bayesian-based lowest common ancestor (BCLA) taxonomic classification software algorithm that has demonstrated more accurate results for species- and subspecies-level classification (17). Another major limitation in this process is the aforementioned issue of primer universality. Different primer pairs target different regions of the 16S rRNA gene sequences, which can result in variable microbiome profiles for the same sample (16). These concerns were confirmed by Hiergeist et al. in an external quality assessment of nine centers utilizing NGS on fecal samples (18). While their study found high intracenter reproducibility, they demonstrated significant differences in the reported relative abundances from the phylum to genus level generated by different centers performed on compositionally identical fecal samples. Thus, the technology is limited in its ability to report accurately on abundance, as the number of read lengths does not always correlate to the relative abundance of each detected species. Although there was significant intercenter variation at all the steps of the complex work flow, Hiergeist et al. found that the greatest variation in the microbiome analysis was driven by the PCR primers used for sequencing and amplification (18). Furthermore, significant differences in microbiome analyses were attributed to the procedures used for data analysis, such as the quality and composition of the 16S rRNA databases utilized, among other factors (18). Another common criticism of nucleic-acid-based analytical methods is their inability to discriminate between the DNA of a viable bacterial cell versus that of a dead cell. However, this problem may be mitigated by the development of molecular viability analyses, including viability PCR and molecular viability testing, which allow for this important distinction to be made in the process of PCR amplification. Additionally, studies have been able to confirm the viability of the sequenced species with the use of EQUC (3, 5). Finally, there is reason to consider lab error in the processing of these tests, where, for example, normal laboratory reagents may cause false-positive detections if used inappropriately.

In addition to identifying components of the microbiome, NGS technology has also been utilized to identify antibiotic resistance genes (ARGs) within a given microbiome, with the intended purpose of tailoring antibiotic therapy and promoting antibiotic stewardship. However, there are also limitations to this application of NGS. Our knowledge of the phenotypic outcomes of ARG expression and interactions in complex bacterial systems, such as the gut or urinary microbiome, is limited. This likely precludes

optimal or safe clinical application of the generated results (19). More specifically, there is a need for further investigation into the characterization of the baseline bacteria and ARGs (both chromosomal and mobile elements) present in the resident microbiome. This would allow us to better understand how ARGs are transferred bidirectionally between the commensal gut microflora and pathogenic species both naturally and under the lens of selective antibiotic pressure (19). Consequently, the “resistome” data obtained through NGS can be used to infer or predict phenotypic antibiotic resistance patterns. However, 16S rRNA gene sequencing alone does not provide the necessary ARG data, thus introducing the added cost and complexity of additional testing with whole-genome sequencing (WGS) or AmpliSeq. Furthermore, NGS is not yet capable of linking genotypic resistance to a specific organism, making it difficult to determine if a reported ARG stems from a commensal or the targeted, pathogenic microbe(s). Therefore, confirmatory standardized growth-based susceptibility testing may still be necessary, as genotypic profiles do not always correlate with the clinical phenotype due to a variety of poorly understood genetic mechanisms. It is imperative that we first better understand the underlying microbiome in order to then maximize the use of antibiotic therapy with low selective pressure on the resident microflora. This ensures that the protective commensal bacteria remain while pathogenic bacteria are eliminated without the transfer or dissemination of resident ARGs.

Expanded quantitative urine culture. EQUUC was developed following discovery of the bladder microbiome in an attempt to grow species that could not be cultivated on standard urine culture. The standard urine culture was designed to detect only the most common, fast-growing, aerobic uropathogens, such as *Escherichia coli*, at a threshold of greater than 1,000 CFU/ml. This has demonstrated a false-negative rate up to 90% (9, 11). Comparatively, EQUUC is able to report bacterial growth as low as 10 CFU/ml, resulting in improved detection of bacteria. Hilt et al. demonstrated that a particular EQUUC protocol can detect up to 92% of species not reported on standard urine culture and that the concordance between cultured genera and sequenced bacterial DNA was as high as 80% (3). EQUUC is done through plating a larger urine sample on a variety of media at different growth temperatures and under a variety of atmospheric conditions, including both aerobic and anaerobic, with a longer incubation time. More specifically, EQUUC generally involves plating 100 μ l of urine on 5% sheep blood (blood agar plate [BAP]), MacConkey, chocolate, as well as colistin-nalidixic acid (CNA) agars. These are incubated under either room atmospheric conditions or a 5% CO₂ incubator at 35°C, and occasionally also 30°C, for 48 h (3, 8). Additionally, 100- μ l samples of urine are plated on CDC anaerobe BAP agar and incubated under both anaerobic conditions and in a Campy gas mixture of 5% O₂, 10% CO₂, and 85% N at 35°C for 48 h (3, 8). This compares to the standard culture technique utilized by most labs, where 1 μ l of urine is plated on BAP and MacConkey agar plates under standard ambient atmospheric, aerobic conditions at 35°C for 24 h (3, 8). Price et al. also describe an “expanded-spectrum” EQUUC protocol, where 3 different volumes of urine (1, 10, and 100 μ l) are plated onto 7 combinations of the above-described agars and environments. They also present the previously mentioned “streamlined” protocol, which involves plating 100 μ l of urine on BAP, MacConkey, and CNA agars in a 5% CO₂ incubator at 35°C for 48 h. This streamlined protocol detected 84% of bacteria found on the expanded-spectrum EQUUC protocol, in comparison to only 33% detected with standard urine culture, which missed most non-*E. coli* pathogens (8).

In the following sections, we will discuss three pathologies most commonly implicated in patients with recurrent or persistent irritative LUTS. These three conditions, including urgency urinary incontinence (UUI), IC, and recurrent UTI, have been studied in the context of the urinary microbiome. They present a potential opportunity for the clinical application of EQUUC and/or NGS.

Overactive bladder/urgency urinary incontinence. Overactive bladder/urgency urinary incontinence (OAB/UUI) is a poorly understood chronic urologic condition experienced by up to 20% of the female and elderly populations. It is theorized to be

the result of abnormal neuromuscular signaling leading to involuntary detrusor contractions, known as overactive bladder. The abnormal signaling and involuntary contractions present as a sudden and strong sensation to void, followed sometimes by leakage of urine (5, 7). However, detrusor muscle overactivity is seen in only approximately 58% of women with UUI, suggesting an alternate etiology exists (7). The diagnostic criteria for UUI have historically included the exclusion of acute UTI given the significant overlap in symptoms of urgency and frequency between the two conditions, based on the previous belief that urine is otherwise sterile. However, more recently, several groups have investigated a potential role of the urinary microbiome in UUI (5–7, 11, 20, 21).

Bacterial DNA has been detected in the urine of 39 to 52% of patients with UUI who do not have clinical evidence of a UTI. Further, patients with UUI and bacterial DNA in their urine had a greater number of mean daily UUI episodes and a lower risk for postinstrumentation UTI compared to patients with UUI and no evidence of urinary bacterial DNA (6, 20). Pearce et al. found that nine genera (*Actinobaculum*, *Actinomyces*, *Aerococcus*, *Arthrobacter*, *Corynebacterium*, *Gardnerella*, *Oligella*, *Staphylococcus*, and *Streptococcus*) were cultured via EQUC more frequently in patients with UUI. They also found that the urinary microbiome of UUI patients had increased proportions of *Gardnerella* and decreased proportions of *Lactobacillus*, with some differences at the species level. However, they found no overall differences in the microbial diversity between patients with UUI and non-UUI patients by NGS, but found increased microbial diversity by EQUC in the UUI cohort (5). Karstens et al. similarly found no difference in the diversity of the urinary microbiome between UUI and non-UUI patients using NGS. However, they did report a significant correlation between diversity and symptom severity in UUI patients, where patients with lower microbial diversity experienced more incontinent episodes than patients with a higher microbial diversity. They also identified 14 bacteria with significant variations in abundances between the two cohorts (7). Conversely, Thomas-White et al. found that the urinary microbiome of patients with UUI was more diverse compared than those of patients without UUI (11). The same group found a relationship between the urinary microbiome and response to oral medication for UUI, where responders had fewer bacteria and a less diverse urinary microbiome in comparison to nonresponders (21). Although results vary, taken together, these data suggest that there is likely a microbial component to UUI. However, due to the discrepancies between findings, further studies are necessary to better elucidate the role of the urinary microbiome in this particular disease state, and subsequently, the exact clinical application of DNA sequencing in this patient population.

Interstitial cystitis. The traditional definition of interstitial cystitis (IC)/painful bladder syndrome (PBS), a chronic pelvic pain disorder more commonly diagnosed in females, is a chronic inflammatory state of the bladder of unknown etiology (12). There are at least four major theories for the pathophysiology of this condition. These include both autoimmune and infectious etiologies, increased bladder wall permeability due to glycosaminoglycan deficiency, and neuropathic inflammation (12). Standard urine cultures routinely fail to implicate an infective agent in patients with IC. However, emerging evidence using DNA-based techniques suggest that the urinary microbiome may be involved in IC. One study that looked at the urine microbiome of patients with IC using DNA sequencing found a decrease in the ecological diversity of microorganisms in IC patients, and a significant increase in the proportion of *Lactobacillus* species compared to healthy controls (12). A different group also found that the microbiome of urine from patients with IC was less diverse and associated with increased levels of inflammatory cytokines (i.e., interleukin-4 [IL-4]), but contained less *Lactobacillus* species than that of matched controls (22). Conversely, two groups have demonstrated that the urinary microbiome may not play a role in this particular pathology. Meriwether et al. looked at the bacterial 16S rRNA from midstream clean catch urinary specimens of premenopausal patients with ($n = 23$) and without ($n = 18$) IC/PBS, as

determined by the O'Leary-Sant questionnaire. They found no significant difference between the two groups when comparing genomic diversity, richness, or evenness (23). Bresler et al. performed a case-controlled prospective study comparing the midstream voided urinary microbiota of 21 women with urinary frequency, urgency, and bladder pain for greater than 6 months with 20 controls. They also found no difference in the bacterial urotypes and diversity between the two groups using both EQUIC and 16S rRNA NGS (24). In light of the controversial findings described, there is still no direct evidence of involvement of bacteria in the etiology of IC/PBS, requiring additional study. Limitations to consider include the inability of 16S NGS to detect eukaryotic microbes, while EQUIC can only identify certain fungi. Furthermore, the discrepancies in the data illustrate the current limitations to the clinical use of this technology and highlight the need for further research before their translation into clinical use. Nonetheless, the use of DNA sequencing techniques and EQUIC in the diagnostic evaluation and management of these patients may eventually further our understanding of the pathophysiology, and ultimately, management of IC.

Recurrent urinary tract infections. Chronic or recurrent LUTS are common in patients suffering from recurrent urinary tract infections (UTIs). Patients presenting with classic clinical symptoms of uncomplicated UTI, including frequency, urgency, and dysuria (in the absence of vaginal discharge or irritation in women), are commonly treated empirically with antibiotics for cystitis without routine urine culture. Those with persistent symptoms or recurrent infections often require additional work up, including a standard urine culture, to determine bacterial strains and antibiotic susceptibilities in order to ensure effective treatment (25). Historically, the gold standard for diagnosis of a UTI has been the standard urine culture with antibiotic sensitivities (C&S). A definitive diagnosis of UTI is classically made when a symptomatic patient's midstream urine culture grows a single bacterial species at greater than 10^5 CFU/ml or 10^3 CFU/ml (25). Furthermore, the 2009 International Clinical Practice Guidelines from the Infectious Diseases Society of America define a catheter-associated UTI with greater than or equal to 10^3 CFU/ml (26).

Acute, uncomplicated cystitis in an immunocompetent, non-childbearing woman is often a clinical diagnosis based on classic signs and symptoms, with *E. coli* as the presumptive uropathogen. It is treated with commonly used agents known to have efficacy against *E. coli*, such as nitrofurantoin, TMP-SMX, or fosfomycin. In this clinical scenario, urine cultures are not indicated or recommended, and prescription of a short course of empirical therapy is both common and appropriate (25, 27). Conversely, the management of patients with complicated UTIs or those with persistent and/or recurrent symptoms warrants laboratory investigation for appropriately tailored management. Nonetheless, many patients with chronic irritative LUTS undergo this diagnostic and therapeutic cycle repeatedly, resulting in increased health care costs, suboptimal patient outcomes, and likely contributing to the growing problem with antibiotic resistance.

One potential reason for the negative urine cultures that are characteristic of patients with LUTS is the possibility that these patients suffer from infections due to atypical or anaerobic bacteria, or bacteria of multiple taxa (polymicrobial infection), rather than the uropathogens commonly tested for on standard urine culture (4, 5, 9, 10, 12). For example, Price et al. demonstrated that standard urine culture missed 88% of non-*E. coli* uropathogens detected by expanded-spectrum EQUIC (8). Pearce et al. and Hilt et al. both used EQUIC to demonstrate that the false-negative rate of the standard urine culture is as high as 90% (3, 5). Another study showed that patients with chronic LUTS harbored a unique spectrum of bacterial species not traditionally associated with acute UTI, which were therefore missed on routine urine cultures. Additionally, these bacteria did not grow on routine culture and were closely associated with shed bladder epithelial cells. This supported prior evidence of the presence of long-term bacterial reservoirs within the urothelial cells lining the urinary tract (4). Furthermore, it has been shown that uropathogenic *E. coli* (UPEC) can evade host innate immune defense

mechanisms by entering bladder urothelial cells and forming biofilms, or pod-like structures made up of bacteria and polysaccharide matrix encased in a uroplakin covering. These biofilms likely contribute to both chronic and persistent infections as they protect bacteria from immune mechanisms such as phagocytosis and antibiotics, while simultaneously promoting mutations and increased resistance. Assuming that an underlying commensal microbiome plays a protective role in urinary health, the overuse or misuse of antibiotics in this setting could be perpetuating the chronic nature of these patients' infections. For example, both Brubaker et al. and Pearce et al. demonstrated a negative association between the presence of urinary bacterial DNA and the risk of postinstrumentation UTI: 10% and 9% of patients with UUI and DNA sequence-positive urine samples developed a UTI postcystoscopy in comparison to 24% and 27% with sequence-negative samples, respectively (6, 20). Additionally, Thomas-White et al. found that day-of-surgery microbiome composition was associated with postoperative UTI risk in patients with pelvic organ prolapse. More specifically, an abundance of *Lactobacillus iners* and depletion of *Enterobacteriaceae* and *Pseudomonas* were protective against postoperative UTI (28).

Finucane voices his concerns for antibiotic misuse in a review of the UTI, emphasizing the ambiguity in defining "urinary tract infection," both clinically and in the laboratory. He suggests "urinary tract dysbiosis" as a more accurate term. Moreover, he presents an eloquent analogy to the respiratory system, pointing out the difference between an upper respiratory tract infection and the more serious pneumonia. The former, likened to an acute uncomplicated cystitis, is both more common and likely to resolve on its own, benefiting primarily from symptomatic management. The latter, similarly to bacteremic bacteriuria or pyelonephritis, requires antimicrobial intervention. Our approach to diagnosis, what constitutes a true infection, and management must change now that we know "everyone is bacteriuric" and as our understanding of the urinary microbiome evolves (29). The use of DNA sequencing techniques and EQUC in both basic science research and the diagnostic evaluation and management of these patients may eventually play a critical role in improving patient outcomes and reducing long-term health care costs.

CURRENT DIAGNOSTIC PRACTICES AND LIMITATIONS

The emerging body of literature surrounding the urinary microbiome and the clinical applications of both NGS and EQUC present an opportunity to improve patient evaluation and management. While developing their streamlined EQUC protocol for improved uropathogen detection, Price et al. looked at symptom scores in a cohort of 75 urogynecology patients who reported symptoms of UTI based on the UTI symptom assessment (UTISA) questionnaire, compared to 75 controls ($n = 150$). They detected a total of 182 uropathogens using their expanded-spectrum EQUC protocol. One hundred ten of these uropathogens were detected in the catheterized urine samples from the UTI cohort, while 72 were found in urine samples from the non-UTI cohort. Standard urine culture grew 33% of the uropathogens detected by EQUC in all subjects, 50% of those found by EQUC in the symptomatic UTI cohort, and only 7% of those found in the non-UTI cohort. Of the 59 UTI patients who completed a UTISA questionnaire following treatment, 41% (24/59) reported the same or worse symptoms following 3 to 7 days of treatment based on standard urine culture results. It was noted that 12 of these patients (50%) had one or more uropathogens missed by standard urine culture, and 13 (54%) had a microorganism of unknown pathogenicity detected by EQUC (8). However, the growth of 72 organisms on EQUC in the control group again calls into question the clinical implications of such testing. Future work focused on the actionability of the results of these technologies is needed.

Taking this a step further, McDonald et al. conducted a comparative study in which 44 patients with acute cystitis were managed based on the results of either standard urine culture or DNA sequencing. Patients were randomized to treatment based on either culture findings (group A, $n = 22$) or DNA testing (group B, $n = 22$). In total, bacteria were identified by DNA testing in all 44 patients, while only 13 patients had

positive standard urine cultures, although the definition of positive culture used in this work was not stated. Consequently, only 7/22 patients in arm A were treated at the outset of diagnosis given positive cultures, while all 22 patients in arm B received immediate antibiotic therapy. The 15 patients in arm A who had negative urine cultures received antibiotic treatment on day 8 based on DNA testing. Improvement in symptom scores was significantly higher in those treated based on DNA testing, with an average 8.5-point improvement on the UTI symptom assessment questionnaire compared to 3.7 in those treated based on standard urine culture (10).

Patient dissatisfaction with, as well as the economic burden of, suboptimal evaluation and management of recurrent UTIs has brought to light several limitations of the standard urine culture, as previously discussed. First, the standard urine culture detects microorganisms at certain thresholds, specifically at greater than 10^3 CFU/ml. This satisfies the definition of 10^5 CFU/ml set in the 1950s by Edward Kass, who was primarily interested in detecting pyelonephritis (30). However, in 1982, Stamm et al. demonstrated clinically significant lower UTI in the presence of 10^2 CFU/ml (9). Thus, no conclusive threshold for bacterial colony units definitive of UTI exists to this day, as evidenced by the variation in guidelines and the more recent findings by Price et al, which suggest that each uropathogen might have its own unique threshold for UTI diagnosis (8). The concept of “contamination” also poses a problem. The traditional practice within the clinical microbiology lab is to consider the growth of two or more species at greater than 10^3 CFU/ml to be indicative of contamination. However, DNA sequencing studies and enhanced culture methods have detected multiple microbes from various species or genera that contain known pathogens, suggesting that the concept of contamination may warrant reconsideration (10, 25). Despite our ability to identify these multiple organisms, their clinical relevance is not fully understood. Indeed, the presence of a bacterial species with the potential for pathogenicity does not imply that the bacterium is responsible for the clinical symptoms. Furthermore, we are currently unable to determine whether organisms identified by NGS are actually from the bladder, or whether they represent potential contamination with skin flora during the urine collection process, which is a possibility when voided, rather than catheterized, samples are used. These limitations hinder the clinical applicability of the results of NGS testing.

Another factor to consider for these various methodologies is the time required by each test. Culture-based methods often require up to 2 to 3 days to generate results. By association and definition, EQUC requires additional time in testing compared to standard culture to generate more comprehensive results. In contrast, companies currently offering DNA NGS provide the results of a quantitative rapid PCR screen of a set panel of commonly implicated microbes within 24 h, followed by a more comprehensive and precise report based on DNA sequencing within 3 to 5 days. However, it is also important to consider the time it takes to send out the specimen, which can be up to a day, and the potential pitfall of improper storage during this process, which can affect the sample's stability and utility. There is potential for the utilization of alternative in-house methods, including nucleic acid amplification tests (NAAT) or matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), which first requires a urine culture, which may overcome some of the current limitations.

These limitations of the standard diagnostic process, as well as the lack of actionable results, may explain the frustrations of practitioners treating patients with persistent or recurrent symptoms impacting their quality of life. However, these frustrations may not be entirely relieved by the results provided by DNA NGS. Both clinicians and patients will need to practice caution and patience as further research elucidates the meaning and clinical implications of the results of DNA NGS and EQUC. Microbiologists and clinicians should work together to advocate for additional funding to support research focused on determining the clinical relevance and implications of results from DNA NGS and EQUC.

CONCLUSIONS

Both of these two new technologies, EQUC and NGS, provide a plethora of information. However, the clinical applicability of these data is unclear as the presence of a microbe does not render it causative of symptoms or necessarily indicate pathogenicity. This may lead to additional frustrations, as clinicians struggle to correctly interpret these results and manage patients appropriately based on their findings. More specifically, it may further exacerbate the clinician's dilemma about appropriate antibiotic prescription practices. Nonetheless, microbiome analysis provides a new avenue for both basic science and translational research aimed at better understanding the human urinary tract in health and disease. Where physicians are currently limited to optimizing symptomatic relief of common disabling symptoms, there is now a new lens through which to study conditions with potentially treatable underlying etiologies. EQUC and NGS are currently the gold standard for scientific research. While the potential to elucidate previously poorly understood pathophysiology also has diagnostic and prognostic implications in the clinical realm, at this time it is largely limited to patients with chronic symptoms and consistently negative standard urine cultures. In the chronic setting, there is both a diagnostic dilemma and the time to pursue additional workup. Conversely, the evaluation of acute LUTS requires a more hastened approach, including urinalysis and empirical antibiotics, followed by standard urine culture-directed tailoring of treatment, as necessary.

The potential clinical applications of both NGS and EQUC could be widespread, with relevance in the pediatric, adult, and elderly populations and in both males and females. The patients most likely to benefit from these tests include individuals with chronic persistent and/or recurrent irritative LUTS. These are patients whose quality of life is severely impacted by symptoms of urinary frequency, urgency, urge urinary incontinence, and/or suprapubic discomfort. Most commonly, these individuals carry a diagnosis of recurrent UTI. However, there is overlapping symptomatology with conditions including OAB/UUI and IC, both diagnoses of exclusion. Through the use of these two techniques, there is potential to rethink the pathophysiology of these conditions and, consequently, the approach to the management of afflicted patients.

As previously described, a patient presenting with symptoms consistent with LUTS is initially evaluated with a urinalysis and urine culture. When these tests come back positive with tangible, actionable results, patients are often treated with empirical antibiotics, followed by tailoring of the antibiotic regimen to target the dominant organism in the context of its antibiotic susceptibility profile. However, the standard diagnostic workup fails those patients who present with clinical symptoms, but have essentially clean urinalyses and negative urine cultures. Given that the urinalysis has suboptimal diagnostic accuracy for UTI, with a specificity anywhere between 41 and 98%, and a negative predictive value of 46 to 96% for leukocyte esterase and/or nitrites, the presence of these two markers, alone or in combination, may therefore not always be required for the diagnosis of UTI, especially in the setting of clinical symptoms (25). These patients are often desperate and looking for answers, rendering this the ideal clinical scenario in which to utilize DNA NGS and/or EQUC. However, clinicians and patients must be aware that difficulty with interpretation of the results is a major limitation to the current clinical use of these tests. Furthermore, patients that receive appropriate antibiotic therapy, but continue to experience either the same or worse symptoms, or those who experience recurrent infections are also good candidates for additional microbial testing. For example, Price et al. recommend using the streamlined EQUC protocol with catheterized urine samples as a supplemental test in patients with clear UTI symptoms but negative standard urine culture, as well as in patients with persistent UTI symptoms (8). In these patients, microbial analysis within a diagnostic framework serves the purpose of thorough evaluation for a treatable infectious etiology prior to additional diagnostic evaluation or alternative therapeutic and/or symptomatic management.

Patients suffering from recurrent UTIs are likely the largest and most heterogeneous

group to benefit from both the diagnostic and prognostic applications of microbiome testing. Recurrent UTIs are frequently diagnosed in young, middle-aged, and post-menopausal females. More often than not, their symptoms are considered idiopathic. In practice, these patients are sometimes placed on a prophylactic antibiotic regimen, and otherwise evaluated and treated frequently in clinic with therapeutic antimicrobials. The optimization and long-term efficacy of this type of management are questionable, especially as it pertains to antibiotic stewardship and the relative harm versus benefit of frequent antimicrobial prophylaxis and/or therapy (27).

The current approach to the patient groups described above is suboptimal. We therefore propose the incorporation of NGS and EQUC in their evaluation. The purpose of microbiome analysis in these patients is two-fold. First, these tests may serve a diagnostic role. As outlined above, the standard diagnostic techniques employed first-line to exclude an infectious etiology have several limitations. The use of either EQUC or DNA NGS in this context is to identify the putative uropathogen or uropathogens and tailor therapy where it is applicable. Furthermore, as technology and our understanding of the urinary microbiome evolve, genetic testing for ARGs and virulence genes will enable further optimization of therapy. EQUC may play a particular role in this regard as it is a growth-dependent test that allows for standard growth-based, phenotypic antimicrobial susceptibility testing. Additionally, in patients who require repetitive evaluation, EQUC may be a more financially feasible option. Finally, in addition to their diagnostic role, EQUC and NGS have the potential to serve as prognostic biomarkers. Patients with susceptibility to chronic or recurrent disease may benefit from regular microbiome analyses to monitor their individual microbial make-up in order to predict exacerbations or flares. Alternatively, in patients with risk factors, or those who are otherwise predisposed to chronic irritative LUTS (i.e., neurogenic bladder population), following the baseline makeup and changes in their urinary microbiome may aid in predicting and/or preventing future pathology. While repetitive use of NGS may be cost-prohibitive, EQUC may be used for monitoring and subsequent diagnostic evaluation once the clinician has an existing knowledge of the patient's baseline microflora.

The use of DNA-based analytical methods has established and characterized the existence of the urinary microbiome. This discovery and the evolving literature challenge our previously held beliefs surrounding the sterility of urine and the pathophysiology of certain conditions affecting the bladder, including recurrent UTI, OAB, IC, and UUI. While limitations to the clinical application of such tools currently exist, they nonetheless hold the potential for advancing our understanding of these conditions and improving their management.

REFERENCES

- Brubaker L, Wolfe AJ. 2017. The female urinary microbiota, urinary health and common urinary disorders. *Ann Transl Med* 5:34. <https://doi.org/10.21037/atm.2016.11.62>.
- Wolfe AJ, Toh E, Shibata N, Rong R, Kenton K, FitzGerald M, Mueller ER, Schreckenberger P, Dong Q, Nelson DE, Brubaker L. 2012. Evidence of uncultivated bacteria in the adult female bladder. *J Clin Microbiol* 50:1376–1383. <https://doi.org/10.1128/JCM.05852-11>.
- Hilt EE, McKinley K, Pearce MM, Rosenfeld AB, Zilliox MJ, Mueller ER, Brubaker L, Gai X, Wolfe AJ, Schreckenberger PC. 2014. Urine is not sterile: use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. *J Clin Microbiol* 52:871–876. <https://doi.org/10.1128/JCM.02876-13>.
- Khasriya R, Sathiananthamoorthy S, Ismail S, Kelsey M, Wilson M, Rohn JL, Malone-Lee J. 2013. Spectrum of bacterial colonization associated with urothelial cells from patients with chronic lower urinary tract symptoms. *J Clin Microbiol* 51:2054–2062. <https://doi.org/10.1128/JCM.03314-12>.
- Pearce MM, Hilt EE, Rosenfeld AB, Zilliox MJ, Thomas-White K, Fok C, Kliethermes S, Schreckenberger PC, Brubaker L, Gai X, Wolfe AJ. 2014. The female urinary microbiome: a comparison of women with and without urgency urinary incontinence. *mBio* 5:e01283-14. <https://doi.org/10.1128/mBio.01283-14>.
- Brubaker L, Nager CW, Richter HE, Visco A, Nygaard I, Barber MD, Schaffer J, Meikle S, Wallace D, Shibata N, Wolfe AJ. 2014. Urinary bacteria in adult women with urgency urinary incontinence. *Int Urogynecol J* 25:1179–1184. <https://doi.org/10.1007/s00192-013-2325-2>.
- Karstens L, Asquith M, Davin S, Stauffer P, Fair D, Gregory WT, Rosenbaum JT, McWeeney SK, Nardos R. 2016. Does the urinary microbiome play a role in urgency urinary incontinence and its severity? *Front Cell Infect Microbiol* 6:78. <https://doi.org/10.3389/fcimb.2016.00078>.
- Price TK, Dune T, Hilt EE, Thomas-White KJ, Kliethermes S, Brincat C, Brubaker L, Wolfe AJ, Mueller ER, Schreckenberger PC. 2016. The clinical urine culture: enhanced techniques improve detection of clinically relevant microorganisms. *J Clin Microbiol* 54:1216–1222. <https://doi.org/10.1128/JCM.00044-16>.
- Brubaker L, Wolfe AJ. 2015. The new world of the urinary microbiota in women. *Am J Obstet Gynecol* 213:644–649. <https://doi.org/10.1016/j.ajog.2015.05.032>.
- McDonald M, Kameh D, Johnson ME, Johansen TEB, Albala D, Mouraviev V. 2017. A head-to-head comparative phase II study of standard urine culture and sensitivity versus DNA next-generation sequencing testing for urinary tract infections. *Rev Urol* 19:213–220. <https://doi.org/10.3909/riu0780>.
- Thomas-White K, Brady M, Wolfe AJ, Mueller ER. 2016. The bladder is not

- sterile: history and current discoveries on the urinary microbiome. *Curr Bladder Dysfunct Rep* 11:18–24. <https://doi.org/10.1007/s11884-016-0345-8>.
12. Siddiqui H, Lagesen K, Nederbragt AJ, Jeansson SL, Jakobsen KS. 2012. Alterations of microbiota in urine from women with interstitial cystitis. *BMC Microbiol* 12:205. <https://doi.org/10.1186/1471-2180-12-205>.
 13. Lewis DA, Brown R, Williams J, White P, Jacobson SK, Marchesi JR, Drake MJ. 2013. The human urinary microbiome; bacterial DNA in voided urine of asymptomatic adults. *Front Cell Infect Microbiol* 3:41. <https://doi.org/10.3389/fcimb.2013.00041>.
 14. Bajic P, Van Kuiken ME, Burge BK, Kirshenbaum EJ, Joyce CJ, Wolfe AJ, Branch JD, Bresler L, Farooq AV. 2018. Male bladder microbiome relates to lower urinary tract symptoms. *Eur Urol Focus* 566:1–7.
 15. Gaitonde S, Malik RD, Zimmern PE. 2019. Financial burden of recurrent urinary tract infections in women: a time-driven activity-based cost analysis. *Urology* 128:47–54. <https://doi.org/10.1016/j.urology.2019.01.031>.
 16. Fuks G, Elgart M, Amir A, Zeisel A, Turnbaugh PJ, Soen Y, Shental N. 2018. Combining 16S rRNA gene variable regions enables high-resolution microbial community profiling. *Microbiome* 6:17. <https://doi.org/10.1186/s40168-017-0396-x>.
 17. Gao X, Lin H, Revanna K, Dong Q. 2017. A Bayesian taxonomic classification method for 16S rRNA gene sequences with improved species-level accuracy. *BMC Bioinformatics* 18:247. <https://doi.org/10.1186/s12859-017-1670-4>.
 18. Hiergeist A, Reischl U, Gessner A. 2016. Multicenter quality assessment of 16S ribosomal DNA-sequencing for microbiome analyses reveals high inter-center variability. *Int J Med Microbiol* 306:334–342. <https://doi.org/10.1016/j.ijmm.2016.03.005>.
 19. Do TT, Tamames J, Stedtfeld RD, Guo X, Murphy S, Tiedje JM, Walsh F. 2018. Antibiotic resistance gene detection in the microbiome context. *Microb Drug Resist* 24:542–546. <https://doi.org/10.1089/mdr.2017.0199>.
 20. Pearce MM, Zilliox MJ, Rosenfeld AB, Thomas-White KJ, Richter HE, Nager CW, Visco AG, Nygaard IE, Barber MD, Schaffer J, Moalli P, Sung VW, Smith AL, Rogers R, Nolen TL, Wallace D, Meikle SF, Gai X, Wolfe AJ, Brubaker L, Pelvic Floor Disorders Network. 2015. The female urinary microbiome in urgency urinary incontinence. *Am J Obstet Gynecol* 213:347.e1–347.e11. <https://doi.org/10.1016/j.ajog.2015.07.009>.
 21. Thomas-White KJ, Hilt EE, Fok C, Pearce MM, Mueller ER, Kliethermes S, Jacobs K, Zilliox MJ, Brincat C, Price TK, Kuffel G, Schreckenberger P, Gai X, Brubaker L, Wolfe AJ. 2016. Incontinence medication response relates to the female urinary microbiota. *Int Urogynecol J* 27:723–733. <https://doi.org/10.1007/s00192-015-2847-x>.
 22. Abernethy MG, Rosenfeld A, White JR, Mueller MG, Lewicky-Gaupp C, Kenton K. 2017. Urinary microbiome and cytokine levels in women with interstitial cystitis. *Obstet Gynecol* 129:500–506. <https://doi.org/10.1097/AOG.0000000000001892>.
 23. Meriwether KV, Lei Z, Singh R, Gaskins J, Hobson DTG, Jala V. 2019. The vaginal and urinary microbiomes in premenopausal women with interstitial cystitis/bladder pain syndrome as compared to unaffected controls: a pilot cross-sectional study. *Front Cell Infect Microbiol* 9:92. <https://doi.org/10.3389/fcimb.2019.00092>.
 24. Bresler L, Price TK, Hilt EE, Joyce C, Fitzgerald CM, Wolfe AJ. 2019. Female lower urinary tract microbiota do not associate with IC/PBS symptoms: a case-controlled study. *Int Urogynecol* <https://doi.org/10.1007/s00192-019-03942-9>.
 25. Wilson ML, Gaido L. 2004. Laboratory diagnosis of urinary tract infections in adult patients. *Clin Infect Dis* 38:1150–1158. <https://doi.org/10.1086/383029>.
 26. Hooton TM, Bradley SF, Cardenas DD, Colgan R, Geerlings SE, Rice JC, Saint S, Schaeffer AJ, Tambayh PA, Tenke P, Nicolle LE. 2010. Diagnosis, prevention, and treatment of catheter-associated urinary tract infection in adults: 2009 International Clinical Practice Guidelines from the Infectious Diseases Society of America. *Clin Infect Dis* 50:625–663. <https://doi.org/10.1086/650482>.
 27. Abbo L, Hooton T. 2014. Antimicrobial stewardship and urinary tract infections. *Antibiotics (Basel)* 3:174–192. <https://doi.org/10.3390/antibiotics3020174>.
 28. Thomas-White KJ, Gao X, Lin H, Fok CS, Ghanayem K, Mueller ER, Dong Q, Brubaker L, Wolfe AJ. 2018. Urinary microbes and postoperative urinary tract infection risk in urogynecologic surgical patients. *Int Urogynecol J* 29:1797–1805. <https://doi.org/10.1007/s00192-018-3767-3>.
 29. Finucane TE. 2017. “Urinary tract infection”—requiem for a heavyweight. *J Am Geriatr Soc* 65:1650–1655. <https://doi.org/10.1111/jgs.14907>.
 30. Kass EH. 1962. Pyelonephritis and bacteriuria: a major problem in preventive medicine. *Ann Intern Med* 56:46–53. <https://doi.org/10.7326/0003-4819-56-1-46>.