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# *An implementation of next generation sequencing for prevention and diagnosis of urinary tract infection in urology*

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**Introduction:** The changing face of current infection phenotypes from planktonic to biofilm type has been developed implicating bacterial biofilms in recurrent infection. To date, no specific medical treatment exists to specifically target biofilms in the human host. Similarly, the identification of a biofilm has relied upon the analysis of tissue samples with electron microscopy or DNA identification with polymerase chain reaction (PCR) and sequencing. Standard culture and sensitivity test is not able to detect a presence of biofilms.

**Materials and methods:** Two types of molecular microbial diagnostic testing “levels” are performed as noted below. In both types of analysis, the microbial DNA is extracted from the patient’s sample. The patient report contains information about the pathogenic bacterial and fungal microorganisms detected, bacterial load and resistance genes to different antibiotics. Once the bacteria have been identified antibiotic recommendations are made based on research confirming the effectiveness of treatment.

*The technique was tested in 112 patients in different areas of urology for prevention and treatment purpose.*

**Results:** The clinical application of next generation sequence in different clinical phase I-II trials (acute cystitis in 56 patients, rectal swabs before transrectal prostate biopsy in 32 men, neurogenic bladder in 13 patients, chronic bacterial prostatitis in 17 men) demonstrated that this novel approach extends our knowledge about the microbiome of the urogenital tract in both men and women. DNA sequence has a high sensitivity to detect a bacterial and fungal association with resistant genes to antibiotics revealed allowing to implement a targeted and individual prevention and treatment of urinary tract infection (UTI) with improved efficacy compared to standard culture and sensitivity technique.

**Conclusion:** The next generation DNA sequence technology enables the discovery of new concepts regarding the role of microorganisms in diseases of the urinary tract with an individualized approach for a more accurate diagnosis, prevention, prophylaxis and treatment of UTI.

**Key Words:** new generation sequencing, urine culture and sensitivity, targeted antibacterial therapy

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## Introduction

There remained a long term dogma that has existed in infectious urology stating, urine of healthy people is sterile.<sup>1</sup> However, many recent studies from several bodies of literature reveal evidence of different microorganisms in the urine of healthy people based on next generation sequencing (NGS).<sup>2</sup> Furthermore, it has been shown that we have an abundance of bacterial cells; many beneficial while others deleterious

representing a unique ecological system. This microbiome can be changed all the time, depending on such environmental factors as diet or the interaction of the surrounding microbiome of other people. Even small alterations to the healthy microbiome can lead to overgrowth of harmful, pathogenic bacteria. This process is referred to as dysbiosis, and it can lead to the development of chronic infection and inflammation.

The changing face of current infection from planktonic to biofilm type has been developed implicating bacterial biofilms in recurrent infection.<sup>3</sup> The discovery of bacteria living in organized communities that compose bacterial biofilms is a revolutionary step forward from the classic understanding of bacterial phenotypes. Traditional teaching encompasses bacterial infections caused by isolated bacteria in planktonic form that are identified by conventional culture techniques quantified by colony-forming units of usually a single bacterial type in a Petri dish diagnosed by inspection. Based on these results, a main pathogen detected was treated with antibiotics, or prevented with specific vaccines. This concept existed for almost one and half centuries providing physicians with limited guidance for tailored treatment. This approach may not be appropriate to explain the increasing role that bacterial infection or colonization is believed to play in human disease. In contrast, bacterial biofilms appear to be relevant in chronic or much more complex infections, which are common manifestations of chronic human illnesses. When in the form of a biofilm, the causative bacteria are difficult to culture and are largely resistant to current antimicrobial therapy. To date, no specific medical treatment exists to specifically target biofilms in the human host. Similarly, the identification of a biofilm has relied upon the analysis of tissue samples with electron microscopy or DNA identification with polymerase chain reaction (PCR) and sequencing. No simple clinical test is available for detecting the presence of biofilms.

Urinary tract infections (UTIs) are the most common type of infection in humans, accounting for approximately 8.6 million visits to health care providers per year and over 1 million hospital admissions in the United States each year.<sup>4</sup> World wide the estimated number of yearly UTIs treated are 150 million and the financial impact of treatment is over \$6 billion US dollars.<sup>5</sup> Many studies have discussed clinical practice guidelines for the treatment of UTI such as cystitis and pyelonephritis. Treatment in many instances of uncomplicated UTIs can be based on empiric antibiotic therapy. In other cases i.e. complicated or recurrent UTIs, therapy cannot be based on laboratory-controlled culture and sensitivity (C&S)

reports. The diagnosis of UTI by clinical criteria alone can have an error rate of up to 33%.<sup>6</sup> Laboratory culture errors may be as high as 50% based on low bacterial loads present in the urine specimen not achieving levels of  $10^5$  or more.<sup>7</sup>

Needless to say, that in the infection process there is a need to properly identify the causal agents in the urine of those who suffer from chronic UTI in timely fashion. Both inaccurate and insufficient therapeutic coverage can lead to increased government and insurance expenditures for the health care of patients with UTI, lost work days and decreased quality of life measures for patients suffering from infections. Therefore, timely and accurate microbial identification is critical for UTI patients. As a rule of thumb, in order to be implemented every new diagnostic method should improve upon the previous one in terms of sensitivity and specificity, positive predictive value and validated in randomized clinical trials. Contrary to the standard culture technique established by Koch and Petri in the 1880s, an introduction of high throughput DNA sequencing (metagenomics) and bioinformatics allowed to analyze complete genomes of microorganisms that led us into “-omics” area.

### *Materials and methods*

Gene sequencing represents a process when a DNA sample is placed in a machine that bathes it with one of the four nucleotides. As the DNA molecules make contact with their complimentary nucleotide, the DNA pieces can be deciphered and assembled into readable code, revealing missing or damaged elements.

The genomic profile of urine bacteria can now be produced rapidly and less costly. This new direction requires a new terminology to understand, Table 1. These “meta-omics” techniques are aimed to target primary sequencing to define a full microbial and fungal association in urine.<sup>8</sup> A priori, the results of genome sequence are superior to functional information from cultured reference bacteria although they need to be proven further. This may help to improve the accuracy of testing and determine which technique can help to decrease patient’s urinary tract symptoms faster with targeted therapy. The improved diagnosis of UTIs may also lead to a decrease in resistant strains of bacteria, which is up to 34% in certain areas of the United States.<sup>9</sup> National and international recommendations warn against the broad use of fluoroquinolones for uncomplicated UTIs and the increasing rates of bacterial resistance worldwide.<sup>10,11</sup> In this context it is now more important that antibiotic therapy be used rationally and specifically in the treatment of UTIs.

TABLE 1. New glossary for high-throughput genomics in urology

Term	Specifics
Microbiome	Characteristic microbial community occupying a reasonable well defined habitat which has distinct physico-chemical properties within their areas of activity, e.g. in urine
Microbiota	Refers to the microorganisms that exist within a niche, e.g. kidney, prostate, bladder etc.
Metagenomics	An ability to analyze multiple gene sequences from large numbers of samples, complemented with functional screening and characterization of randomly cloned DNA fragments e.g. from urine
Metatranscriptomics	Uses high-throughput sequencing or microarray analysis to examine RNA expressed e.g. in urine samples, thus targeting bacteria that are transcriptionally active
Metaproteomics	Performs protein separation and sequencing to define the major proteins present in urine
Metabonomics	Is not directly related to genetic information of the microbes with acting machinery (cell-mediated effects and direct identification to a target microbial species), but describes the metabolite profile resulting from total microbial activity in the urine. This analysis can provide a direct metrics of the consequences of the microbial activity in urine

**DNA sequence testing**

Each patient’s urine/ semen/ rectal swab samples were shipped to MicroGenDX laboratory in Lubbock, TX, USA using FedEx overnight courier service.

Two types of molecular microbial diagnostic testing “levels” are performed as noted below. In both types of analysis, the microbial DNA is extracted from the patient’s sample. The patient report contains information about the pathogenic organisms detected.

Once the bacteria have been identified antibiotic recommendations are made based on research confirming the effectiveness of treatment. The reference used for antibiotic treatment in the MicroGenDX laboratory is the Johns Hopkins antimicrobial guidelines reference 2015-2016 based on NGS comprehensive DNA sequencing analysis of virtually the entire universe of microorganisms (more than 25,000 species), Figure 1.

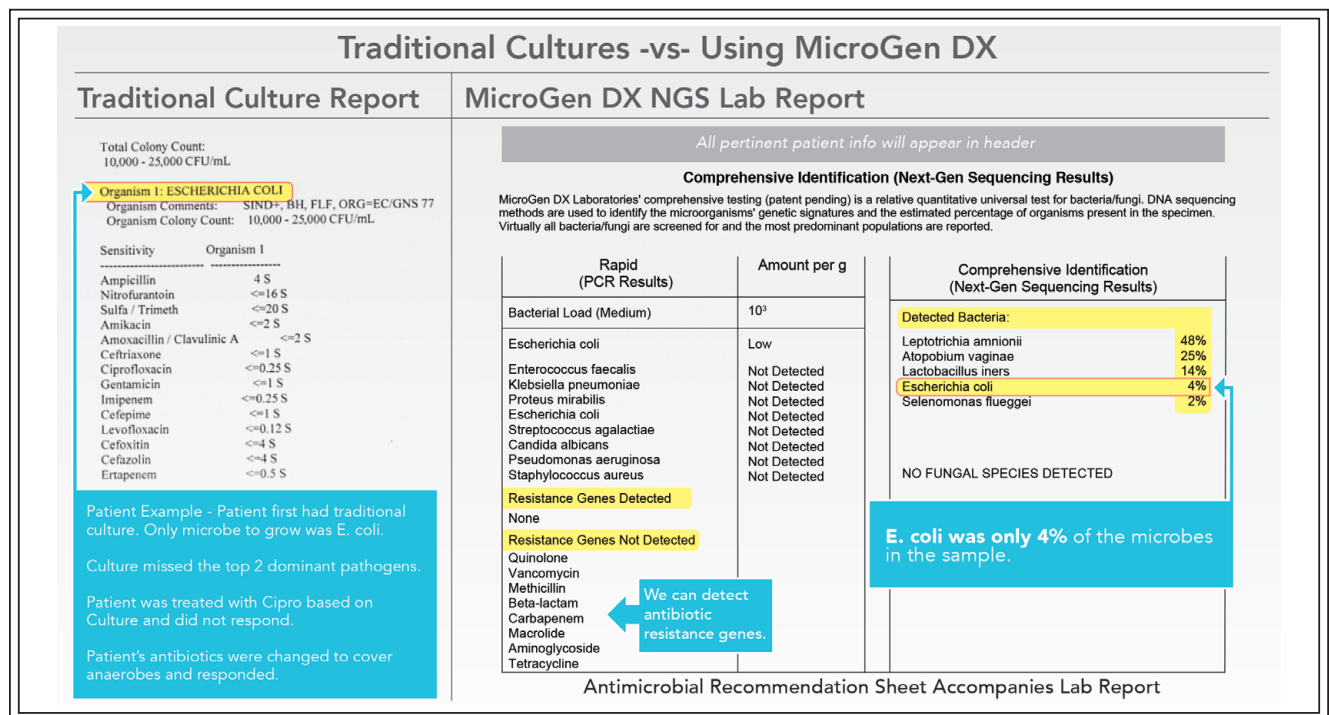


Figure 1. The comparative reading of the traditional culture versus DNA sequencing.

*Level 1 – Rapid screen using quantitative PCR method for bacteria and fungi*

The Panel utilizes unique genes present in each organism to identify how much of that organism is present in each patient sample. This concentration is achieved in a multistep process. The 16S universal bacteria were a semi-quantitative assay, which reports the total bacteria load as low, medium, or high. These ranges for general bacteria copy number per mL or mg are < 10<sup>5</sup>, 10<sup>5</sup>-10<sup>7</sup>, and > 10<sup>7</sup> for low, medium, and high respectively. The sensitivity and specificity were 100% on *E. faecalis*, *E. faecium*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. pyogenes*, *vanA*, *C. albicans*, *E. coli*, *P. mirabilis* and *S. pneumoniae*. The sensitivity and specificity for *S. agalactiae* were 100% and 99.4%. The sensitivity and specificity for *mecA* are 100% and 95.2% and for 16S are 100% and 97.1%.

The Panel also includes a qualitative real-time PCR test for resistance genes responsible for eight main families of antibiotics such as vancomycin, methicillin, beta-lactams, carbapenems, macrolides, aminoglycosides, and tetracyclines. The resistance genes profile was performed on a qualitative assay, which was reported them out as present or absent.

With one material sample company provides the results within 2-3 days including overnight shipping if patient has antibiotic resistance and eight common microbes. It allows starting initial targeted antibacterial therapy.

*Level 2 – DecodEX*

DNA pyrosequencing method detected virtually all microbial organisms and fungal pathogens that may

be present in patient specimens. Microbial DNA in each sample was sequenced using the Ion Torrent PGM sequencer in order to establish what type of bacterial and fungal species are present. This assay is capable of detecting the organisms listed here from as little as 100 ng of patient specimen. Every species can be accurately detected with as little as 10 ng/μL of extracted DNA with at least 1000 copies of each species. Each organism is reported as a relative abundance in the sample.

A total of 180 American Type Culture Collection (ATCC) species were run on bacteria and fungi sequencing assays to confirm accuracy, sensitivity, and specificity. All ATCC species matched their intended targets. Ten fungi samples were detected on the bacterial assay; however, the cultures were confirmed contaminated using an alternate sequencing platform (Roche 454 sequencer). Both assays produced 100% accuracy, sensitivity, and specificity.

*Antibiotic susceptibility*

The database used for comparison of DNA amplicon from entire 16S database analysis has been curated to eliminate incomplete entries and known mistakes in entry. MicroGenDX laboratory has used the information gained from the analysis of over 100,000 samples to understand the circumstances from which urologists can get multiple potential species identified as probable results (ties) and have used multiple techniques to help us determine the most probable result in these circumstances, Figure 2.

The determination of the bacterial species, including genetic resistance targets, provides for a susceptibility determination, which clinicians can base upon

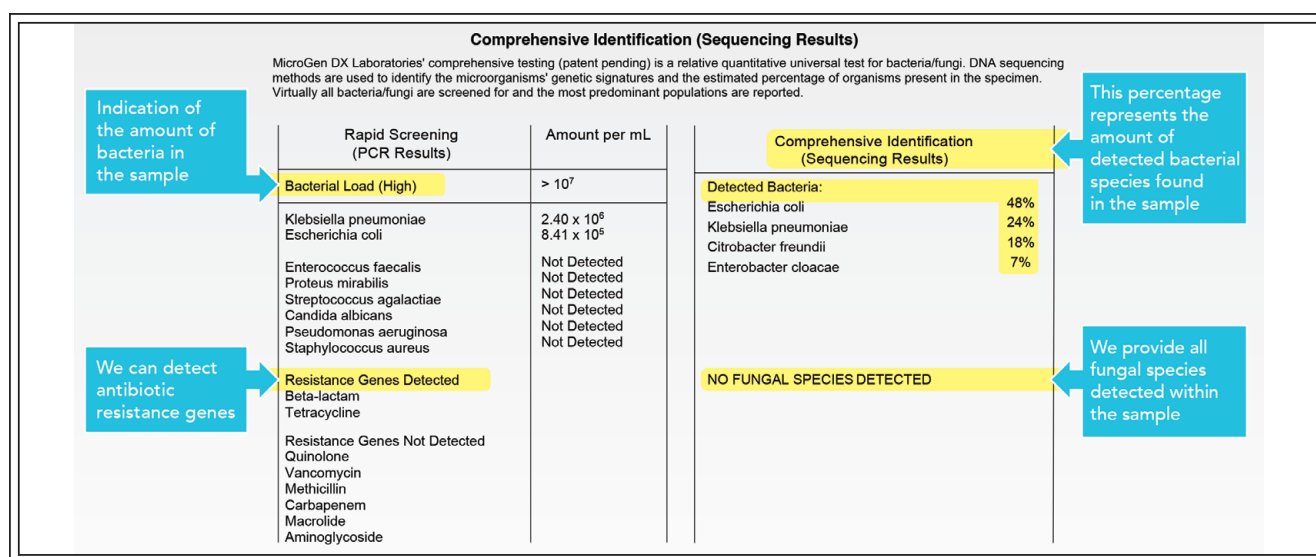


Figure 2. Breakdown results of level 1 and 2 DNA sequencing.

local antibiograms and/or clinical references. The laboratory employs the antibiotic susceptibility database from the Johns Hopkins Antimicrobial Guideline database, which is compiled from current best practice literature references to identify the most common antibiotics to treat the identified microbes. Ultimately, the laboratory deliberately recommends antibiotics that are not subject to mobile genetic element issues (e.g., mec A cassette, ESBL, van) and that are as specific to each patient as possible. Finally, the results of comprehensive DNA sequencing analysis of virtually the entire universe of microorganisms is being provided in 3-5 business days. This analysis can facilitate an adjusted targeted therapy.

The clinical testing of DNA sequence were performed in 112 patients with acute UTI (cystitis), bacterial prostatitis, chronic UTI due to neurogenic bladder with urinary retention, and in patients before transrectal prostate biopsy for prevention of severe UTI. The different samples were used as urine, semen and rectal swabs from these patients. The studies were approved by Institutional Review Board of the Florida Hospital.

## Results

### *A head to head comparative phase II study of standard urine culture and sensitivity versus DNA next generation sequencing testing for UTIs*

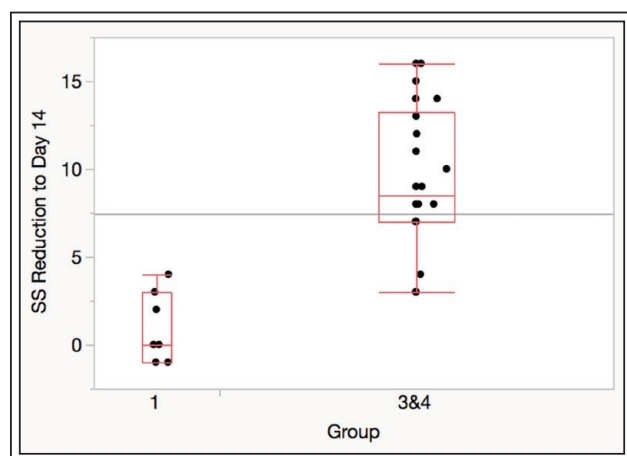
Between January 2016 and December 2016, 56 patients were entered into this study with symptoms of a UTI and 44 patients completed the study.<sup>12</sup> Twenty-two volunteers were entered in a control group without symptoms of a

UTI. Based on a head to head comparison, symptom scores were significantly better for those patients whose treatment was based on DNA results versus traditional culture studies. All 44 patients showed positive results in DNA sequencing tests, while only 13/44 patients had positive urine culture tests. In the cohort of patients where treatment was based on culture results with culture negative and DNA positive results treatment outcomes were improved with respect to symptom scores when they started treatment on day 8, Figure 3. Another possible advantage of the DNA test is an increased sensitivity to diagnose anaerobic flora noted in 20/44 patients with symptoms of a UTI.

### *Individual targeted prophylaxis of severe UTI prior transrectal prostate biopsy*

The primary aim of this study was to evaluate a value of NGS of rectal swabs prior to transrectal prostate biopsy in order to perform individual prophylaxis for each patient. The 32 patients enrolled into this study were followed for any infectious complication within 30 days after the biopsy. Standard protocol for empiric prevention of infection included 2 days of levofloxacin 0.5 g before the biopsy and 1 g ceftriaxone with adjustment for targeted prophylaxis in each case.

Summarizing phase I study on 32 patients, multiple microbial species were revealed with median nine organisms (range: 2-16), Table 2. The predominant flora was found to be E. Coli – in 11 men, Bacteroides – in 9, Prevotella – in 4, Citrobacter - in 2; Corynebacterium- in 2, Klebsiella, Campylobacter, Fenollaria and



**Figure 3.** Symptom severity reduction at day 14 in treatment arms. Two-sample t-test p value is  $< 0.001$ .<sup>12</sup> Reprinted with permission of MedReviews®, LLC. *Rev Urol* 2017;19(4):213-220. All rights reserved.

**TABLE 2. The dominant bacteria in rectal swabs**

Bacteria	Patients
Esherichia coli dorei	11
Bacteroides dorei, fragilis, vulgaris	9
Prevotella copri	4
Citrobacter koseri & freudii	2
Corynebacterium striatum, aurimucosum	2
Klebsiella pneumonia	1
Campylobacter hominis	1
Fenollaria timonensis	1
Faecalibacterium prausnitzii	1
Multidrug resistant	20 (incl. 18- quinolones)

TABLE 3. The dominant fungi in rectal swabs

Fungi	Patients
Candida albicans, glabrata, dubliniensis & zeylanoides	7
Aspergillus tubingens	1
Saccharomyces cerevisiae	2
Multifungal association	6

Faecallobacterium in 1 patient, respectively. In 20 of 32 cases multidrug resistance genes were detected, and 18 of those 20- to fluoroquinolones. It allowed us to change our empiric prophylaxis in 18/32 (56.3%) to an antibiotic other than levofloxacin. In 10 cases' different fungal species were detected 6 of which were multifungal that we considered as an indication to add antifungal medications, Table 3. The targeted prophylaxis based on these results allowed avoidance of severe infectious complications in all 32 patients within 30 days after biopsy.

### Chronic bacterial prostatitis (CBP)

A retrospective review of the semen DNA NGS results of 17 patients with symptoms of chronic CBP (NIH category II) was performed. All fresh semen specimens were collected from patients and processed through the whole bacterial and fungal semen microbiota spectrum. All 17 patients had positive DNA NGS results. The median number of microorganisms present in each specimen was of 4.4 species, range (1-10). Resistance genes to different antibiotics detected were found in 4/17 specimens. Nine of the 17 semen samples had primarily gram positive bacteria mostly from the Enterococcus family, 6/17 had mixed and 2/17 primarily gram-negative pathogens. In two cases a high bacterial load ( $> 10^7$  microorganisms) was revealed, in 4- a moderate load ( $10^6$ - $10^7$ ), and in 11/17 - a low load ( $\leq 10^5$ ), respectively. Two patients had fungal species in association with bacterial pathogens.

While the diagnosis and treatment of CBP represents a difficult task for practicing urologists this new technology of DNA NGS may provide a new diagnostic tool to analyze complete genomes of microorganisms in a timely manner and implement targeted and individual treatment of CBP. A significant number of patients had gram positive organisms representing mostly Enterococcus family, which may lead us to revisit our approach to the treatment paradigms for CBP in prospective studies.

### Neurogenic bladder (NGB)

The urine NGS results of 13 patients with NGB associated with chronic urinary retention was analyzed in a retrospective study to monitor levels of bacteriuria and fungiuria to prevent flare-up episodes of chronic UTIs. The median age of patients was 45 (range: 25-79). The cohort of patients' included NGB secondary to stroke (4), spina bifida (3), dementia (3), spinal cord injury or surgery (2) and cerebral palsy (1). The urine diversion was performed via clean intermittent self-catheterization (CISC) (6 cases), indwelling Foley catheter (4), suprapubic tube (2) and Mitrofanoff CIC (1). The urine samples were obtained from all patients.

All 13 patients had positive NGS results. The median number of organisms present in each specimen was of 3 species, range (1-9). The majority of patients (10) had a high bacterial load ( $> 10^7$  microorganisms), 1 - had a moderate load ( $10^6$ - $10^7$  microorganisms), 1- had a low load ( $10^5$  or less microorganisms) and 1- did not have bacteria, only an association of 5 fungal species. Resistance genes to different antibiotics detected were found in 9/13 samples and 6 of them had multidrug resistances genes. The dominant microorganism was found as E. Coli (5 cases), Staph aureus, saprophyticus and hominis (3), Enterobacter hormachei (1), Hemophilus parainfluenzae (1), Pseudomonas aeruginosa (1), Klebsiella oxytoca (1). Two patients had fungal pathogens and one of them in association with bacterial pathogens.

The treatment of NGB remains a challenging and difficult area in urology practice especially defining an optimal preventive or treatment plan for chronic UTI. This new diagnostic tool such as NGS can provide clinicians with complete information on individual genomic profile of microorganisms for regular monitoring of UTI in order to prevent and treat in a targeted manner. A significant number of patients had multidrug (including quinolone) resistant E. Coli, that needs to be readjusted moving from empiric to targeted antibacterial therapy.

### Discussion

Recently, the NGS or metagenomics sequencing has provided a new approach for the detection of microorganism that have been non-culturable by standard culture and sensitivity techniques in the urinary microbiome. A very broad spectrum of bacteria and fungi was detected in the "sterile" bladder urine in healthy individuals as well as in patients with different urological disorders. Nowadays it takes at least 24 hours to detect a bacterial load and genes resistant to main groups of antibiotics to start an antibacterial therapy.

In 2-3 days the comprehensive results of metagenomics sequencing becomes available to re-adjust a targeted anti-bacterial and/or fungal treatment.

An implementation of culture-independent metagenomic sequencing (NGS) enables us to detect a wide range of fastidious, anaerobic and even non-culturable bacteria.

We should embrace these new technologies in order to revisit and redefine UTIs and various urological disorders based on findings by routine culture and sensitivity only. The NGS will shed new light on the poorly understood etiological relationship of microorganisms and host in urine/rectal swabs/semen etc. microbiome.

The urologic community expects that the wide introduction of novel NGS technologies will further improve diagnostic findings, as the significance of sequencing data is based on the number of sequencing reads.

First, in spite of an increasing application of NGS into routine urologic practice, some limitations should be mentioned which could obscure all benefits of the technique. Having a high sensitivity for detection of all microbial and fungal species makes it difficult to interpret the results of sequencing. Even an exact quantitative distribution of all microbial associations, may make it more difficult to define which bug or superbug can cause a UTI. The presence of a dominant microorganism does not suggest necessarily that truly one can be more dangerous than second bacteria with less percentage distribution. On the contrary, in some situations some commensal microflora is an integral part of a complex of natural mechanisms on mucosal surfaces of urinary tract infections that could be considered as a safeguard for the resistance of the organism against pathogenic microorganisms. Therefore, we have to define an algorithm of detection for truly dangerous pathogens that needs to be treated.

Secondly, another limitation for the choice of targeted antibiotic(s) is an indirect antibiogram based on the results of 25,000 species done before. Together with resistance gene information this may help when choosing the right antibiotic(s) although additional validation needs to be done for different urogenital tract diseases.

Third, regarding cost/efficacy ratio it will probably take some time before novel NGS assays become clinically affordable by health systems and patients. However, the sequencing related costs have decreased rapidly and the cost for urine/semen or rectal swabs is \$199 to process through MicroGenDX.

Fourth, recent data from the body of literature has shown that results from different trials obtained by

different protocols and laboratories are variable thus lacking reproducibility and reference data. The DNA sequencing protocols also can be different regarding the implemented DNA extraction platform, PCR primers, and bioinformatics approach of downstream of DNA sequencing. In other words, there is an unmet need for standardization and consensus on interpretation of received data.

Fifth, the majority of studies on urinary microbiome have included a collection of mid-stream urine samples into a sterile container or through the insertion of a urethral catheter. It makes the technique extremely susceptible to the contamination of the sample with pathogens inhabiting the distal urethra and even skin around urethral meatus and possible vaginal flora in women.

## Conclusion

The clinical application of NGS in different clinical phase I-II trials demonstrated that this novel approach extends our knowledge about the microbiome of the urogenital tract in both men and women. The technology enables the discovery of new concepts. Regarding the role of microorganisms in diseases of the urinary tract with an individualized approach for a more accurate diagnosis and subsequent treatment.

However, as we have learned regarding the example of asymptomatic bacteriuria, the pure detection of bacteria in the urine does not prove an infectious etiology in a specific disease. To better understand the shifting paradigm of current urinary infections where bacteria may play a role we need to integrate NGS in well-designed clinical studies overcoming the above-mentioned limitations. It is also of the utmost importance that implementation of a new NGS methods will lead to the decreased consumption of antibiotics with more targeted and shorter term action preventing growing resistance and side effects.

## Disclosure

Both authors Dr. Vladimir Mouraviev and Dr. Michael McDonald are the clinical research consultants for MicroGenDX. □

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