

Pregledni rad | Review paper

Genetic and genomic (molecular) diagnostic methods in identifying the cause of urinary tract infections and urinary microbiota

Genetičke i genomske (molekularne) dijagnostičke metode u identifikaciji uzročnika infekcija mokraćnog sustava i sastavnica urinarne mikrobiote

Andrea Janeš^{*1}, Marina Čepnja^{*2,3}

¹ Department for Clinical Microbiology and Hospital Infections, Clinical Hospital Sveti Duh, Zagreb, Croatia

² Special Hospital Agram, Polyclinic Zagreb, Biochemical Laboratory, Zagreb, Croatia

³ Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia

*both authors contributed equally

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✉ Corresponding author:

Andrea Janeš
Sveti Duh 64, 10 000 Zagreb, Croatia
janes.andrea.mef@gmail.com

Summary

Standard urine culture (SUC) is a widespread diagnostic test. According to various guidelines, SUC is not a recommended first-line diagnostic tool for uncomplicated urinary tract infections (UTIs). However, its results are valuable in patients with complicated UTIs, hospitalized patients, and those who failed empirical antibiotic therapy. The emergence of antibiotic resistance has reaffirmed the urine culture as the gold standard in UTI workup. Nevertheless, turn-around-time and cost-effectiveness of SUC are the main incentives for continuous exploration of new, faster, and more sensitive procedures for evaluating the count and species of microorganisms and their susceptibility to antibiotics. Therefore, we considered it important to write a review that analyzes the advantages and disadvantages of state-of-the-art UTI diagnostics. We aim to compare standard cultivation methods with diagnostic modalities based on multiplex PCR, 16S RNA genes sequencing, and next-generation sequencing suitable for analyzing whole urinary microbiomes.

Sažetak

Standardna urinokultura česta je dijagnostička pretraga, no prema smjernicama se ne preporučuje u obradi nekomplikiranih infekcija mokraćnog sustava, već je indicirana za bolesnike s kompliciranim infekcijama mokraćnog sustava, hospitalizirane bolesnike i one kod kojih empirijska antibiotska terapija nije polučila klinički odgovor. Pojava bakterijske rezistencije na antibiotike naglašava važnost urinokulture s antibiogramom kao zlatnog standarda u obradi infekcija mokraćnog sustava. Molekularne dijagnostičke metode kao što su multipleks PCR, sekvenciranje 16S RNA gena i sekvenciranje nove generacije, prikladne su za detekciju urinarnih patogena i analizu urinarnog mikrobioma, a zbog brzine i osjetljivosti nalaze svoje mjesto u kliničkoj obradi urinarnih infekcija. Cilj ovog rada je usporediti prednosti i nedostatke suvremenih metoda dijagnosticiranja infekcija mokraćnog sustava s klasičnim kultivacijskim metodama.

Introduction

Although still considered the gold standard, conventional urine culture is time-consuming and limited in scope.^[1,2] Therefore, it is understandable that medical and scientific communities are searching for a faster, more accurate, and affordable method with high clinical utility. Molecular gene analysis techniques such as polymerase chain reaction (PCR) or genome analysis such as targeted gene or complete genome next-generation sequencing (NGS) are being researched to fulfil those needs. PCR is widely used

in targeted diagnostics; NGS of 16S ribosomal RNA (rRNA) gene amplification can determine bacterial genera and their abundance but is unsuitable for in-depth species analysis. At the same time, metagenomics NGS is the most comprehensive and unbiased method.^[3,4,5] Randomized controlled trials (RTC), which could evaluate how the clinical utility of PCR or NGS compares to standard diagnostics, are lacking.^[5] Nevertheless, PCR is already widely implemented in clinical microbiology laboratories, while NGS slowly permeates from academic and reference laboratories

into the clinical setting.^[6,7] With hand-held size sequencers on the market now, NGS might become an attractive option for an average clinical microbiology laboratory.^[7] This review aims to analyze the available gene and genome analysis methods, their advantages and disadvantages in UTI diagnostics, and compare them with standard cultivation methods.

Methods

Citations for this article were acquired by searching English scientific literature in the Web of Science database. The search was conducted using the following keywords: urinary tract infection, UTI, urine culture, polymerase chain reaction, PCR, sequencing, next-generation sequencing, and NGS. The total number of articles recovered was 436. Of those, 401 article was discarded due to duplicates, unrelated subject, epidemiological and phylogenetic studies, molecular methods used on samples other than urine, basic science studies, veterinary studies, case reports, molecular methods used on cultured uropathogens as the means of detecting their virulence factors or resistance genes, and molecular methods used as the means of detecting rare and specific uropathogens in urine such as *Burkholderia mallei* and *pseudomallei*, and *Mycobacterium tuberculosis* complex. The final number of articles for review was 35.

Usefulness of PCR in clinical practice and comparison with other methods

PCR-based diagnostic testing has become widely available in UTI work-up. Using multiple primers to detect several targets simultaneously significantly reduces the cost and turn-around time of the findings and significantly increases the clinical usefulness of the tests. Although multiplex PCR is still not routinely used as the first-line diagnostic method in the context of UTIs, numerous studies have compared PCR results with standard urine culture (SUC). Multiplex PCR can be used directly on urine samples and shorten the time to results, making it a valuable diagnostic option. A study by Wojno et al.^[1] compared multiplex PCR applied directly to urine samples with a SUC in the elderly male population (median age 77 years, range 60-95 years). Their research was conducted on 582 voided and catheterized unique urine samples of patients suspected to have a UTI as judged by a urologist. Significant bacteriuria consistent with UTI was detected in 56% (326/582) of samples when PCR was used, while the standard urine culture detected bacteriuria in only 37% (217/582) of samples.^[1] PCR and culture agreed in 74%, with both being positive in

34% (196/582) and both negative in 40% (282/582). In 22% of samples, PCR was positive while culture was negative, and in 4% (21/582) of samples, culture was positive while PCR was negative. Concordance between PCR and culture was 90% when both methods yielded results, which agrees with previous studies.^[12] It was debated whether the high sensitivity of PCR revealed true uropathogens, which for whatever reason did not grow in culture, or if it detects bacterial contamination at a much higher rate. Heytens et al.^[8] compared voided urine samples of 220 symptomatic women (mean age of 38,5 years) who complained of dysuria and/or increased urinary urgency and 86 asymptomatic women (mean age of 37.2 years). The study found that 95.9% of patients in the symptomatic group were qPCR (quantitative PCR) positive for *E. coli*.^[8] At the same time, urine cultures in the symptomatic group yielded *E. coli* or other uropathogens in 80.9% of samples.^[8] A difference of almost 15% more proven UTI infections with qPCR than with the standard urine culture was observed. In the asymptomatic group, there were 10.5% positive samples by culture and 11.6% by PCR.^[8] If the more significant proportion of the positive results in the symptomatic group were due only to the detection of bacterial contamination, we would also expect to observe this effect in the asymptomatic group. Therefore, these results highly suggest that PCR is more likely to detect true pathogens, which do not represent contamination or a non-significant result caused by the higher sensitivity of PCR.^[8,3] It is clear that standard urine culture is negative in 25-30% of patients with clinical presentation of UTI and pyuria.^[8] This might be because the majority of the bacterial population is confined to intracellular biofilms and, therefore, not readily grown in culture. Another possibility is that, in some cases, anaerobes and other fastidious bacteria are the major UTI pathogens that cannot grow in a standard urine culture.^[3,9] In the study of Wojno et al.^[1], PCR was negative and culture positive only in instances of bacterial species not included in the PCR panel. This fact indicates that the scope of the multiplex PCR panel limits its sensitivity.^[1,2] Polymicrobial results pose a significant challenge for clinical interpretation. In the study of Wojno et al.^[1], 95% of those were detected by PCR, while only 22% by culture.^[1] With polymicrobial samples, there is always a question of the clinical significance of the results and the specific pathogenic contribution of every single detected pathogen because, even with quantification of bacteria, it is not always clear if the most abundant bacterial species detected by PCR or NGS is the causative agent of UTI. These questions remain unresolved, especially with implementing ever more sensitive methods in detecting bacteriuria.

In addition, the quality of samples is of utmost importance, with freshly catheterized urine being a more acceptable sample than midstream urine. The clinical interpretation of polymicrobial results is ever challenging, especially in the context of our growing knowledge concerning the urinary microbiome and its possible role in the etiology of UTIs. Another set of studies has also compared the performance of multiplex PCR and SUC on urine samples with different bacterial counts.^[1] Even though PCR has a higher detection rate of single pathogens and is more efficient in detecting multiple pathogens compared with SUC, a serious disadvantage of this method and the difficulty of meta-analyzing the results lie in the fact that multiplex PCR is used exclusively in the form of predefined target panels. Hence, the results depend on the choice and the combination of target primers.

Urinary microbiota and methods of its examination

It is still unclear what comprises a healthy urinary microbiome in terms of specific bacterial species and their overall diversity.^[3,5] With the discovery of the urinary microbiome, there has been a considerable shift in the UTI pathogenesis paradigm. Since „everyone is deemed bacteriuric” by NGS, the pathophysiology of UTI is now explained by urinary microbiome dysbiosis rather than by the intrusion of bacteria into a sterile space.^[5] It is possible that chronic urinary tract conditions, now considered idiopathic or non-infectious, might have specific microbiome profiles, contributing to clinical presentation and acute worsening of symptoms.^[5,10] Whether the composition of „normal” urinary microbiota has a protective, detrimental or neutral role in the pathogenic course of UTIs is still the focus of research. As is the case with microbiota in other niches, the composition of urinary microbiota differs significantly between individuals, and we still do not have a description of typical urinary microbiota. SUC used to be an excellent test for detecting UTIs when subscribing to the paradigm of urine normally being sterile. Apart from sterile cultures, all other results were then deemed abnormal. As the paradigm has shifted and now it is established that urine is not sterile but has a healthy microbiome, the limitations of SUC are more evident. The big problem is the liberal threshold of what constitutes a positive result on SUC, which depends on pre-analysis (e.g., sampling method) and clinical presentation (e.g., lower or upper UTI). Also, high contamination rates and a high rate of false negative culture results have called into question the reliability of the current gold standard.

NGS is the most commonly used method in the examination of urinary microbiota. If it is ultimately proven that there are wide varieties of a healthy urinary microbiome, it might be helpful to define and measure specific markers of inflammation in addition to microbiome analysis. A study by Cheng et al.^[11] developed precisely such a method: they used metagenomics NGS to evaluate the microbiome in clinical urine samples and the host’s cell-free DNA (cfDNA).^[11] By measuring the host’s cfDNA, they evaluated the level of inflammation and the host’s tissue injury.^[11] They found that patients with bacterial UTI had higher cfDNA counts corresponding to neutrophils and bladder cells, indicating the recruitment of inflammatory cells to the site of infection and consequent tissue damage.^[11] In bacterial UTIs, these markers of inflammation are relatively straightforward and can easily be detected by standard urinalysis and urine sediment inspection. However, a subset of urology patients might benefit from such a dual metagenomic approach. Those are patients with kidney transplants.^[11] They often suffer from viral kidney infections not detected by standard culture or even multiplex PCR panels that capture the usual bacterial urinary pathogens. Using this approach, one can detect these infections with precision and measure their degree, even before any inflammatory changes are revealed in the kidney biopsy samples. Not to mention that the need for kidney biopsy might be obviated altogether if this method proves to have higher sensitivity than biopsy for detecting nephropathy in such cases.^[11]

Possibilities and variants of NGS in the study of urinary microbiota and diagnosis of UTI

UTI diagnostics are likely to advance significantly as genomic sequencing propels innovation. As mentioned earlier, the problem of negative urine culture in patients with clinical presentation of UTI and pyuria remains. It might be that the cause of these symptoms originates from the biofilm or from specific species of microorganisms not being able to grow under SUC conditions, so these questions open a space for new diagnostic methods such as NGS. The great advantage of this method is being culture-independent.^[12] The scope of sequencing can be divided into three primary groups: genome sequencing, transcriptome sequencing or targeted sequencing. Sequencing of specific marker genes (i.e., targeted sequencing), such as the prokaryotic 16S rRNA genes, is most commonly used. 16S rRNA genes sequencing targets a highly conserved region with nine hypervariable regions, which allows further speciation among species, and is a well-established method for comparing sample phylogeny and taxonomy in

complex microbiomes. As a high-throughput method, 16S rRNA gene sequencing is used more often than the long-lasting and comprehensive NGS based on whole genome sequencing.

Despite its slow turn-around time, shotgun metagenomic sequencing allows for a simultaneous study of all genes of all organisms present in complex samples. This methodology also provides insight into unculturable microorganisms that are otherwise difficult to analyze. Today, we consider NGS a superior method with 100% accuracy - 100% sensitivity, and 95% specificity in bacterial identification.^[13] Despite the incredible power of the method to identify microorganisms that urine culture cannot, the question of the results' clinical significance in UTI patients remains. The NGS results state the overall bacterial load expressed in DNA copies per mL, and the abundance of a single microorganism is compared and expressed as a proportion relative to the overall bacterial load. Comparing such results to colony-forming units per millilitre (CFU/mL) on SUC is complex. In addition, the species composition is significantly different from the species most commonly detected by SUC: with SUC being biased toward aerobes and NGS revealing a more prominent contribution of anaerobes than previously thought.

A severe limitation of NGS technology is the quality of the genomic reference libraries. The databases are public and often uncurated, leading to incorrect entries of sequences and genomes. It follows that it is impossible to ensure quality control without accurate reference materials.^[3] For this reason, we believe significant changes in this segment are needed to interpret the results accurately. Despite these shortcomings, the method is indispensable in examining the urinary microbiota and setting reference standards for a healthy urinary microbiome.^[14] There is hope that the NGS methodology will lead to revolutionary progress in UTI diagnostics.

Comparing different UTI diagnostics methods

The main problem in diagnosing UTIs nowadays using standard urinary culture is its relatively high rate of false-negative results. In the past, such cases of negative urine culture, especially in otherwise healthy young women who experience UTI symptoms, were considered a consequence of a „urethral syndrome” or even psychosomatic.^[3] Nowadays, it is known that urethral syndrome does not exist as a clinical entity, and by implementing methods such as PCR and NGS, it has become clear that those patients have significant bacteriuria, albeit undetected by the conventional culture.^[8,3,14,2]

Many bacterial species that NGS can detect are also cultivable using enhanced quantitative urine culture (EQUC).^[3] However, EQUC is a laborious process for an average clinical microbiology laboratory, and, most importantly, it does not significantly shorten the time for obtaining results, and speedy results would be useful for initiating targeted antibiotic therapy. NGS can be slow in that regard, too. It is estimated that the time to results is about 3-5 days.^[5,7] Another important drawback of NGS and PCR, which might be resolved in the future, is their inability to inform clinicians about the true phenotypic antimicrobial resistance profile of the relevant pathogens.^[7,5,3] Although it can successfully detect antibiotic resistance genes, it cannot distinguish whether these are present in residential flora or the pathogens.^[3,5,7] In addition, the presence of the resistance genes does not correlate well with the phenotypic resistance.^[3,5,7,15] Therefore, standard antibiotic susceptibility testing based on cultivation is still a golden standard. However, there are ambitious plans and developments of an NGS-based method that might accurately predict the resistance phenotype and give a minimal inhibitory concentration (MIC) for an antibiotic of interest.^[7]

One instance in which NGS results of antibiotic resistance genes proved useful despite the controversies described above is outlined in a study by Mouraviev et al.^[16] It included 68 patients who were about to undergo transrectal prostate biopsy and needed preoperative antimicrobial prophylaxis. Routinely they would have all received levofloxacin. Rectal swabs were taken, and NGS was performed. In 47% of patients, fluoroquinolone resistance genes were detected, among other types of resistance genes. NGS also detected fungal species in 27 cases; therefore, an antifungal agent was added to the prophylaxis regiment. In conclusion, patients who received personalized prophylaxis based on NGS results had no serious infectious complications 30 days post-surgery. There was one case of cystitis and two of epididymitis.^[16] To reiterate the downsides of NGS, these are the following: costs; uncurated libraries with incorrect annotations; a non-existent or underdeveloped system of external quality control and proficiency testing; general lack of validation and regulation, at least when applied to the field of microbiology; and the need to have staff educated in bioinformatics and big data analyses, or the need to outsource those analyses to external bioinformatic firms.^[5,3,7] In addition, the clinical utility of NGS results is still not definitively proven, i.e., it is not clear whether those results significantly affect the outcome of patients with UTIs.

To our knowledge, only one study compared urine culture and NGS in terms of patients' outcomes. Uri-

nary frequency, urgency, dysuria, and abdominal discomfort were among the acute cystitis symptoms in the group under consideration, along with potential haematuria symptoms. Patients were allocated into groups with simple and complex acute cystitis. Fifty-six participants with acute cystitis symptoms were enrolled in the study; 12 people were excluded based on protocol requirements approved by the IRB; 44 patients finished the study. The control group included 22 asymptomatic individuals. The results showed that when culture-negative patients with UTI symptoms had undergone NGS urine testing and received antibiotic therapy based on NGS findings, their UTI symptoms significantly subsided.^[17] However, more research is needed to evaluate the utility of NGS diagnostics for clinical decisions concerning individual patients and, more globally, antimicrobial stewardship programs. As of this writing, NGS results are still not used effectively for antimicrobial stewardship.^[7,5]

For the NGS results to be clinically relevant, it would be helpful for clinicians to have a point of reference to know what a healthy urinary microbiome

looks like in terms of the variety of bacterial species and their abundance.^[3] Otherwise, NGS results might lead to unnecessary treatment.

Additionally, the problem of thresholds remains, and those must be clearly outlined in any future guidelines concerning NGS-based UTI diagnostics.^[3] In diagnosing UTIs by conventional culture, it is accepted that every pathogen has its corresponding cut-off value to meet microbiological criteria for infection. Therefore, the accepted threshold for *E. coli* is $\geq 10^2$ CFU/mL of urine, while for other uropathogens, it is $\geq 10^4$ CFU/mL.^[18] Quantification is also possible when quantitative PCR (qPCR) is used.^[8] Units in qPCR are genome equivalents (geq) per mL, and they correspond well to CFU/mL.^[8] In NGS, the units are the number of reads.^[10] The accepted clinical thresholds are still somewhat arbitrary and depend on the implicated pathogens, method of sampling, and the presumed site of infection within the urinary tract; therefore, the problem of the clinical utility of UTI thresholds is not going to be entirely resolved with more sensitive diagnostics; on the contrary, it might be amplified by it.

TABLE 1. COMPARISON OF CURRENT URINARY TRACT INFECTIONS DIAGNOSTIC METHODS

TABLICA 1. USPOREDBA SUVREMENIH METODA DIJAGNOSTIKE INFEKCIJA MOKRAČNOG SUSTAVA

| | SUC | EQUC | PCR | NGS |
|------------------------|--|---|--|--|
| Utility | Golden standard for the diagnosis of acute UTI. | Mostly confined to research setting. Patients with UTI symptoms and negative SUC. Patients unresponsive to current antimicrobial therapy when other causes are excluded. | Used in conjunction with SUC. Clinically indicated in cases of UTI symptoms and negative SUC or to detect pathogens not readily recovered by SUC. | Mostly confined to research setting. Reports on urinary microbiome. May be clinically useful in specific group of patients (e.g., kidney transplantation) and those with chronic urinary tract conditions (both infectious and non-infectious). |
| Time to results | 2-4 days for bacteria (longer for fungi). | 2-4 days (for aerobic bacteria – longer for anaerobes or fungi). | 1 day 1-2 days for Pooled Antibiotic Susceptibility Testing (P-AST™, Guidance UTITM, Pathnostics). | 3-5 days |
| Downsides | Time to results Accuracy in diagnosing UTI diminishes in heavily colonized patients (chronically ill, elderly, patients with indwelling catheters). ^[19] | Urine sampling via bladder catheterization preferred. Additional costs compared to SUC.* Time to results. Able to detect wider range of microorganisms, but cannot differentiate UTI vs non-UTI (requires clinical input). | Dependence on the panel of selected primers. Additional costs compared to SUC.* Usually no report on phenotypic AST, only on resistance genes. | No consensus on what comprises a healthy urinary microbiome. Relative, not absolute, quantification of microorganisms. No report on phenotypic AST (yet). Dependence on the quality of genomic reference libraries. Need for skilled personnel. Big data storage. Cost.* Time to results. |

| | SUC | EQUC | PCR | NGS |
|--|---|--|--|--|
| Antimicrobial sensitivity testing (AST) | Detects and reports phenotypic resistance. | Detects and reports phenotypic resistance. | Mostly detects and reports only on resistance genes. Pooled Antibiotic Susceptibility Testing (P-AST™, Guidance UTI™, Pathnostics). | Detects and reports on resistance genes. |
| Accuracy | 95% sensitivity and 85% specificity for diagnosing UTI when the threshold is set at 10 ² CFU/mL. Accuracy of diagnosing UTI influenced by the set threshold (10 ⁵ vs. 10 ² CFU/mL) and the population of interest (healthy women vs. disabled elderly individuals with indwelling catheters). | In one study ^[20] SUC vs. EQUC: SUC detected only 33% (60/182) of all uropathogens detected by EQUC. Pathogens detected both in UTI and non-UTI cohort, therefore clinical interpretation required. | In one study ¹ of patients with urinary tract symptoms, PCR and culture agreed in 74% of cases (431/582). Both positive 34% (196/582), both negative 40% (235/582), PCR positive, culture negative 22% (130/582), PCR negative, culture positive 4% (21/582). | 99.2% accuracy, listing species by relative abundance. ^[21] Excellent at detecting microorganisms, but meta-analyses on clinical significance lacking. |

*Upfront cost of the diagnostic method itself may be higher than SUC, but the total patient's healthcare cost may be decreased due to timely and/or in-depth results.

Conclusion

Although PCR and NGS are growing more available in UTI workup, SUC remains the gold standard. There are several reasons for that. First, SUC is standardized, i.e., cut-off concentrations of bacteria are agreed upon, depending on the bacterial genera, the method used to obtain urine (clean-catch midstream, bladder catheterization, suprapubic puncture), and the presumed site of UTI within the urinary tract. Furthermore, although constrained by relatively long time-to-results, during which healthcare costs and adverse outcomes due to inadequate therapy might occur, SUC still holds a significant advantage: the ability to provide a clinician with antimicrobial susceptibility profile, which in turn guides the selection of appropriate antimicrobial therapy, infection control measures, and antimicrobial stewardship efforts.

In terms of detecting microorganisms in a urine sample, NGS metagenomics is the most robust method, but its strength over multiplex PCR and 16S rRNA gene sequencing lies not merely in its higher sensitivity but in the fact that the method is unbiased. Although valuable and affordable, PCR platforms can detect only „expected” pathogens and already familiar antimicrobial resistance mutations. On the other hand, NGS detects all pathogens included in a reference base, which is hundreds more than the number of pathogens included in a multiplex PCR panel. NGS also detects all known resistance genes, as well as their novel mutations. With NGS, one gathers an incredible amount of

information, most of which one still has trouble interpreting. In addition, no reference point for a healthy human urinary microbiome is fully defined and clinically verified. Currently, multiplex PCR and NGS are not used as stand-alone methods but almost always as an adjunct to SUC, with NGS still mainly confined to research settings.

When thinking about clinical utility and cost-effectiveness, the balance can probably be achieved by implementing stepwise protocols and carefully selecting patients who might benefit from the multiplex PCR approach and those who might be candidates for more in-depth analyses such as NGS. For example, it is reasonable to try EQUC or the multiplex PCR approach for patients who suffer from recurrent UTIs but repeatedly have negative urine cultures. NGS could be used as the next step if the EQUC or multiplex PCR failed to yield conclusive results. When evaluated for urinary tract infection and nephropathy, kidney transplant patients would probably benefit from the metagenomics approach. On the other hand, if proven that flares of chronic conditions, such as urinary incontinence/overactive bladder, interstitial cystitis, or neurogenic bladder, might be caused by microbiome imbalance, it would be justified to monitor those patients by NGS and treat the flares accordingly. Tailoring preoperative prophylaxis for specific urologic procedures with direct PCR-based antibiotic resistance genes screening or NGS results might prove to be cost-effective and another step in the direction of personalized medi-

cine. Moreover, if NGS-based MIC detection becomes a reality, clinicians would have a robust method with a confirmed clinical utility at their disposal that would affect not only the treatment of a single patient but also inform antimicrobial stewardship programs and infection control measures.

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