Review

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Microbiome Analysis Using Next-Generation Sequencing in Urinary Tract Infections

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In recent decades, the understanding of the genetic information of microbes and hosts has advanced considerably with the development of next-generation sequencing (NGS). For infectious diseases, genomic analysis can provide valuable information on the host disease susceptibility, microbial pathogenicity, and drug sensitivity. For urinary tract infections (UTI), NGS can reveal the pathogenic microbe and the dysbiosis of the urinary microbiome, which is a crucial factor in the pathogenesis of UTI and other urinary tract disorders. This review outlines the role of urinary microbiome dysbiosis in UTI, urinary stone disease, and cancer. Furthermore, the recent advances in NGS technologies for future applications in infectious disease research are described in detail.

Keywords: High-throughput nucleotide sequencing; Microbiota; Urinary tract infections

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INTRODUCTION

The recent COVID-19 pandemic has highlighted the importance of host and microbial genetics in infectious diseases. For the host, variants of the innate immunity genes, such as toll-like receptors or interferon-alpha receptors, are significantly associated with severe symptoms and mortality [1-3]. Rapid genomic evolution of the virus has also been observed, which increases infectivity and avoids immune surveillance [4]. Similarly, in urinary tract infections (UTIs), innate immunity plays a significant role in the early host response to bacterial invasion [5]. Therefore, variants of the toll-like receptor genes, chemokine genes, and interferon response genes are associated with the host susceptibility to UTI and the symptom severity [5-8]. Furthermore, urinary tract microbes, such as uropathogenic *Escherichia coli* (UPEC), have evolved by mutating their proteins or adopting exogenous genes, adhesins (pili and curli), toxins (lipopolysaccharide and hemolysin), and iron acquisition systems (siderophore and hem receptor), as well as undergoing immune evasion (capsule, o-antigen, cellulose) [9]. On the other hand, bacterial strains, such as *E. coli* 83972, have evolved to promote symbiosis by decreasing the virulence factors (hemolysin) and increasing the colonization factors (adhesins) [10]. These bacteria usually do not lead to the development of urinary symptoms [10] and may even provide protection against recurrent UTIs [11,12].

Currently, urine culture is the primary method for identifying pathogenic microbes. On the other hand, not all microbes (bacteria, fungi, or viruses) can be cultured using standard techniques (low sensitivity) [13]. Moreover, the bacteria cultured from the urine of an asymptomatic host may lead to unnecessary or potentially harmful antibiotic treatments (low specificity) [14]. Indeed, the presence of

UPEC alone in UTIs does not always correlate with the development of urinary symptoms [15]. Next-generation sequencing (NGS) of urine samples showed that the microbiome or an aggregate of diverse microorganisms exists, even in the urine samples of "culture-negative" UTIs [16,17]. Increasing evidence suggests that a disruption of the urine microbiome, or "dysbiosis" of the urinary tract microbes, can result in infection and other urinary tract disorders [18-21].

Microbiome analysis offers new avenues for basic scientific and translational research to understand the human urinary tract in health and disease states better. Clinically, potential applications include individuals with chronic recurrent lower urinary tract symptoms. NGS may help better understand the pathophysiology of these conditions and help develop approaches to managing patients who suffer. This review first outlines the role of urinary microbiome dysbiosis in UTIs. NGS is the primary tool to assess the microbiome that has evolved rapidly in the last decade [22-24]. Therefore, this paper describes in detail the recent developments of NGS technologies for potential applications in microbiome research.

MAIN BODY

1. Urinary Microbiome and Urinary Tract Infections

The findings of symbiotic urinary bacteria and the application of NGS technologies for urinary microbe analysis have shown that urine is not sterile but carries normal urinary flora [25-27]. Similar to the normal intestinal flora that protects the bowel from colonization by exogenous pathogenic bacteria [28], the "urinary microbiome" is closely related to the development of UTIs [29-31]. For example, the urinary microbiome is affected by the host genetics, age, diet, and comorbidities, all of which have been clinically implicated in recurrent UTIs [32,33]. Even in otherwise healthy hosts, the urinary microbiome can differ according to age and sex. Fredsgaard et al. [34] used bacterial 16S rRNA amplicon sequencing to analyze the bacterial DNA in clean-catch midstream urine samples from prepubertal hosts. They reported that the urinary microbiota of prepubertal children is different from that of healthy adults, and there was a significant difference in the urinary microbiota between young boys and girls [34].

Recurrent UTI often occurs due to a persistent bacterial population residing in the urinary tract or close body sites—the vagina or the intestinal tract [35]. NGS has helped identify recurrent bacteria and their genetic characteristics in samples from patients with UTIs [36]. In particular, the chronic use of low-dose or full-dose antibiotics can affect the urinary microbiome significantly, leading the urinary tract to an infection-prone state [37-40]. Mulder et al. [37] performed 16S ribosomal RNA (rRNA) sequencing of urine samples from 27 elderly participants who received antimicrobial drugs. They reported that the use of antimicrobial drugs affects the alpha-diversity of the genitourinary microbiota, which may persist in the long term and lead to recurrent UTIs. Rani et al. [39] also showed that kidney transplant recipients who received prophylactic trimethoprim-sulfamethoxazole treatment had an "infection-prone" urine microbiome different from those of healthy adults: decreased microbial diversity and increased abundance of potentially pathogenic bacterial species, such as *Enterococcus faecalis* and *E. coli*. This is important because the use of prophylactic antibiotics, such as trimethoprim-sulfamethoxazole, may not be able to eradicate the pathogenic bacteria [41]. This is in line with the issue of treating asymptomatic bacteriuria, which may indeed be helpful in preventing UTI [42,43].

2. Next-Generation Sequencing Tools for Microbiome Analysis

Culture is the current standard for assessing bacteria in the urinary tract, which harbors several limitations, such as an inability to detect slow-growing anaerobic bacteria. Moreover, as discussed above, information regarding the imbalance in the microbial community, or "dysbiosis" may be more scientifically and clinically relevant than culture positivity. In this regard, NGS has several advantages. 1) It provides a bird's-eye view of the urinary microbiome, and 2) it is possible to avoid culture and species isolation processes that are time and labor consuming, which has, in turn, enabled a dynamic, large-scale comprehensive analysis. Lastly, 3) it helps detect difficult-to-culture microbes, particularly when combined with enhanced urine culture techniques [29]. This paper reviews the current and future NGS techniques for urinary microbiome analysis.

1) Amplicon sequencing vs. metagenomics

Currently, amplicon sequencing is the most commonly

used method for microbiome analysis. The 16S rRNA gene has been most commonly used for urinary microbiome analysis. The 16S rRNA gene comprises nine hypervariable segments (V1-V9) flanked by highly conserved regions. Amplicon sequencing is based on the fact that a universal polymerase chain reaction (PCR) primer can target these highly conserved regions across diverse species to amplify the gene in the sample. After amplifying the 16S rRNA gene conserved region, the attached variable regions are sequenced to gather information regarding the presence/abundance of specific microorganism taxa, or "operational taxonomic units" (OTUs). The OTU counts refer to the relative abundances of each organism in the analyzed sample. In addition, the bioinformatics analysis pipeline allows the construction of phylogenetic trees from representative sequences of OTUs and downstream statistical analyses. Nevertheless, a few disadvantages exist. In general, sequencing one or two of the nine hypervariable regions is only sufficient to achieve taxonomic classification at the family or genus level (the bacterial community can be classified by multiple taxonomic levels: domain > phylum > class > order > family > genus > species). Furthermore, the pre-amplification step by PCR could lead to bias in the abundance–presence results. Metagenomic sequencing is an option if higher taxonomic resolution and functional information are required. Because metagenomics methods sequence the full genome, it results in much more extensive data than amplicon sequencing, which also affects the speed and difficulty of downstream analyses.

(1) Use of amplicon sequencing in urinary microbiome research: Human urine is considered a sample of the low-biomass bacterial community, which can affect the quality of microbiota analysis results significantly [44,45]. Amplicon sequencing targeting bacterial-specific genomic sites reduces unnecessarily abundant host (human) DNA and helps accurately assess the urinary microbiota. Moreover, the sample collection and storage conditions can affect the results. Bundgaard-Nielsen et al. [46] investigated whether the collection and storage conditions influenced the urinary microbial composition. They found no day-to-day variations in the urinary microbiota composition. Furthermore, samples stored at -80℃ and -20℃, but not 4℃, were comparable to freshly handled voided urine.

Vesicoureteral reflux (VUR) is a risk factor for recurrent UTIs and a deterioration of the renal function. Vitko et al. [47] performed 16S rRNA sequencing of urine samples from VUR patients. In VUR patients, *Dorea* and *Escherichia* were dominant, and *Prevotella* and *Lactobacillus* were depleted. Furthermore, the microbial composition varied according to the recurrent febrile UTI status, suggesting the pathological remodeling of urinary bacterial communities after UTIs.

Ureteral stent encrustation is a rare but severe complication. Bacterial film formation is a likely cause of initiating stent encrustation. Kait et al. [48] profiled the microbiota of patients with indwelling ureteral stents using 16S rRNA amplicon sequencing. They collected the ureteral stent, cut the distal end bladder tip, and extracted DNA together with separately collected midstream urine. They used sequence variant (SV) count tables instead of OTUs, which provide improved accuracy in terms of the taxonomic classification and are directly comparable across different studies. The stent microbiota is stable and reproducible, and the most abundant SVs were the bacterial genera, *Staphylococcus*, *Enterococcus*, *Lactobacillus*, and *Escherichia*. Contrary to common beliefs, antibiotics use and having no prior history of UTI were not correlated with the stent microbiota, but were associated with the patients' comorbidities. This suggests that prophylactic antibiotics are not enough to prevent ureteral stent-associated complications, such as bacterial infection and encrustation [48].

(2) Metagenomics sequencing in urinary microbiome research: 16S rRNA amplicon sequencing is often limited to family and genus level resolution, presenting challenges to species-level identification. Metagenomics reads sequences from entire genomes within the genetic pool, which provides higher taxonomic resolution and the detection of non-bacterial species and functional levels, such as the presence of multidrug-resistance genes within the microbial community [49,50]. In healthy subjects, bacteriophages and human papillomaviruses are found frequently in urinary virome analysis [51]. In immunocompromised hosts, such as organ transplantation patients, the viral etiology serves as a significant fraction of the urinary tract and systemic infectious complications [50]. Moustafa et al. [52] used metagenomic sequencing to detect *Ureaplasma*, *Candida*, or *Trichomonas vaginalis*, as well as viruses, such as herpes virus, human papillomavirus, or polyomavirus, all of which were undetectable by 16S rRNA

amplicon sequencing. Furthermore, paired metagenomic sequencing of the urine from transplant recipients and donors revealed the frequent occurrence of the JC polyomavirus in samples from donors and transmission to their corresponding recipients [53]. This suggests that conventional diagnostic tools for virus detection do not cover the full spectrum of the urinary virome. In another study, in kidney transplant recipients with or without bacterial/viral UTIs, urinary cell-free DNA metagenomics sequencing uncovered the presence of bacteria and viruses that were not detected using conventional diagnostic protocols [54].

2) Short-read vs. long-read sequencing in microbiome analysis

Recently, "newer" generation sequencing devices have been developed and rapidly adapted to the field of clinical and basic genomic research. In particular, "long-read" sequencing techniques commercialized by Pacific Biosciences or Oxford Nanopore have shown potential in microbiome analysis. Despite the relatively less accurate base-calling ability, the "long-read" sequencers generate 10,000-100,000 bp reads, which are 10-100 times larger than the current standard "short-read" sequencers, providing 200-500 bp. This is beneficial for assigning each read to specific bacterial taxa [55]. For 16S rRNA gene sequencing, long-read sequencing (LRS) reads the full-length 16S rRNA gene at once (the target 16S gene is approximately 1,500 bp in length), providing a direct representation of the taxa. By contrast, short read sequencing identifies smaller segments of the hypervariable regions (e.g., V1-2, V3-5, or V6-9).

Although there are no reports of applying long-read 16S rRNA or metagenomics sequencing in the urinary microbiome analysis, the results from non-urogenital microbiota studies may help guide future studies [23,24,56]. Wei et al. [57] compared LRS and short-read sequencing (SRS) technologies for conventional 16S rRNA sequencing (V3-V4 reads) of genomic DNA from fecal samples. The two tools provided similar classification results at the genus or species level. Matsuo et al. [55] tested LRS and SRS for sequencing the full-length 16S rRNA gene amplicon of mock and human fecal samples. Compared to the V3-V4 region, the full-length data of the hypervariable regions provided significantly improved accuracy for taxa classification. When LRS full-length and SRS full-length were compared, they provided similar results at the taxa level, LRS, and SRS. At the species level, however, LRS showed better resolution for certain species identification [55,58]. Nevertheless, in the other species, such as *Bacillus* or *Escherichia*, a sequence of LRS or SRS could not provide species-level resolution information owing to the inter-species similarity in their full-length 16S gene sequences [59].

Metagenomics provides a closer representation of the bacterial community diversity and dynamics than amplicon sequencing. While short-read data performs well in classifying bacterial communities, long-read data can enhance the accuracy in identifying non-bacterial or newly discovered bacterial species [60,61]. This is partly because of the nature of input DNA, where SRS uses short, regularly fragmented nucleotides, whereas LRS uses long native DNA or RNA [62]. This often leads to a complete single-contig genome of bacterial species, which is smaller than the average contig length of the LRS data [63]. Despite this, there are only a few papers reporting long-read metagenomics. Driscol et al. [64] performed a shotgun metagenomics analysis using the Pacific Bioscience platform. They reported three complete bacterial genome data of previously unknown species from a co-culture from a lake. The conventional SRS approach (Illumina) resulted in an approximately 7% gap in the genomes, where many essential and functional genes were located. SRS could not identify their structure because these regions were filled with repetitive sequences. Warwick-Dugdale et al. [65] utilized another LRS platform, "Oxford nanopore MinION", for viral metagenomics. Unlike the bacterial community, many viral species lack reference genomes, and there is no established "universal" gene marker, such as 16S rRNA for bacteria for a low-resolution surveillance study. LRS can help distinguish the origin of viral nucleotides between similar strains and assemble a viral genome from a complex community. Compared to the SRS approach, LRS provides a more complete assembly of the viral genome, captures the microdiversity of the viral population, and is more cost-effective. Irinyi et al. [60] utilized the MinION to characterize the fungal community, mycobiome. They aimed to identify *Pneumocystis jirovecii*, which could be identified only by direct microscopic examination or real-time quantitative PCR because of its difficult culturing. LRS metagenomics revealed the presence of *P. jirovecii* and the associated mycobiome, which included *Aspergillus* or *Cryptococcus*, which could become a source

of secondary, recurrent infections. For the bacteriophage community, where most genomic data are incomplete, Kiguchi et al. [66] reported that LRS technology helped detect fragmentations in the phage genomic data from SRS, particularly near repeat sequences and hypervariable regions. Yahara et al. [67] also studied the "oral phageome" using LRS metagenomics and observed several new complete assemblies of viral genomes, as well as the interaction of the phages and the host bacteria [68].

CONCLUSIONS

Understanding the microbial diversity and interactions with the host immune system is crucial for properly managing UTIs. Historically, a urine sample that shows no growth in the standard culture technique was considered "sterile". On the other hand, the recent advances in sequencing technologies revealed the existence of a urinary microbiome. Furthermore, urinary microbiome dysbiosis is associated with infections and lower urinary tract symptoms, urolithiasis, and cancer. Considerable heterogeneity of the urinary microbiome exists across otherwise healthy hosts related to age, sex, prior medications, or non-urogenital systemic conditions. There are several technical challenges in the genomic analysis of the urine microbiome, including bias from DNA primers, amplification, sample contamination, or simply insufficient background genetic information about the microbes. Newer generation sequencers, such as LRS, may help rebuild the microbial genome and detect clinical and biological significant variations.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTIONS

H.H. and J.Y.L. participated in data collection, designed the study, and wrote the manuscript. Both authors read and approved the final manuscript.

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