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Review Article

Advancing Clinical Microbiology: Applications and Future of Next-Generation Sequencing

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Abstract: Next-generation sequencing (NGS) has reformed infectious disease management, including COVID-19. While real-time polymerase chain reaction (PCR) is widely used for rapid pathogen detection, it requires predefined targets. NGS offers an unbiased approach, detecting multiple pathogens simultaneously without prior knowledge. Despite its potential, NGS implementation in clinical settings faces challenges like high costs and technical complexity. NGS platforms like Illumina, Ion Torrent, and Nanopore provide high-throughput sequencing, identifying pathogens and resistance markers. Applications include whole genome sequencing (WGS), metagenomic NGS (mNGS), and targeted NGS (tNGS). Integrating NGS with conventional methods could improve diagnostics, but current evidence is mixed for supporting its widespread clinical use.

Keywords: Next-Generation Sequencing (NGS), Infectious Disease Management, Pathogen Detection, Metagenomic Sequencing, Whole Genome Sequencing (WGS), Laboratory Diagnostics, Molecular Diagnostic Techniques.

INTRODUCTION

Next-generation sequencing (NGS) technology has revolutionized our approach to managing outbreaks, such as the COVID-19 pandemic, and accumulating evidence supports its clinical applications for infectious diseases [1, 2]. Over the past decade, molecular diagnostic techniques have experienced significant advancements, becoming increasingly integral to clinical microbiology laboratories. These techniques facilitate the rapid detection of nucleic acids from pathogens in clinical specimens without the need for culture-based growth. Among these, real-time polymerase chain reaction (PCR) is the most prevalent, amplifying pathogen-specific nucleic acids to enable highly sensitive and specific detection and quantification of a pathogen's genetic material. PCR-based diagnostics have further evolved into multiplex assays capable of simultaneously detecting multiple pathogens [3, 4]. However, these assays are limited to identifying predefined targets, requiring prior knowledge of the suspected pathogens or genetic targets for effective detection.

In contrast, NGS-based tests offer the potential for an unbiased diagnostic approach that allows for the simultaneous and comprehensive detection of multiple pathogens directly from patient samples [5]. This agnostic method does not necessitate prior knowledge of the target organisms, presenting a significant advantage over traditional PCR methods. Despite this promise, the widespread clinical implementation of NGS-based diagnostics faces substantial challenges. Addressing these challenges is crucial to fully realizing the transformative potential of NGS in infectious disease detection and management.

A Brief Overview of NGS

Over the past three decades, numerous NGS platforms have been developed, enabling high-throughput, massively parallel sequencing of thousands to billions of DNA fragments. This represents a significant advancement over first-generation Sanger sequencing, which produces single DNA sequences and is traditionally employed for identifying unknown microbes in clinical samples or for detecting mutations in known genes [6]. However, Sanger sequencing often presents interpretive challenges when applied to complex

or polymicrobial samples, thus it is generally reserved for pure microbial isolates or clinical specimens that are typically sterile.

A key advantage of NGS over PCR is its ability to detect and identify pathogens without the need for prior knowledge of the target organisms or specific primers. NGS can simultaneously generate sequences for numerous pathogens within a single sequencing run, enabling the reliable identification of multiple organisms in a single specimen [5]. Recent advancements have significantly reduced both the cost and complexity of NGS instrumentation, enhancing its suitability for clinical applications. A comparative summary of molecular diagnostic approaches for infectious diseases is presented in Table 1.

In NGS, genomic material from a clinical specimen or isolate is fragmented, randomly amplified, and used to create a library of genomic fragments that are subsequently sequenced [7]. The sequencing methodology varies across different NGS platforms. Two widely used platforms are Illumina and Ion Torrent. Illumina employs sequencing by synthesis, where a fluorescence signal is generated upon nucleotide incorporation. In contrast, Ion Torrent sequencers detect pH changes that occur during nucleotide incorporation. The signals produced—whether from fluorescence or pH changes—are independently and simultaneously recorded for each genomic fragment and translated into nucleotide sequences (A, C, G, or T). These sequenced fragments are then assembled using bioinformatics software, either with the aid of a reference sequence or through de novo assembly.

Nanopore technology has emerged as a highly promising sequencing platform [8]. Distinct from Illumina and Ion Torrent, Nanopore sequencing involves the analysis of single-stranded DNA that can span up to several hundred thousand base pairs without requiring active DNA synthesis. This method records changes in ionic current as the DNA strand traverses a protein nanopore, subsequently translating these signals into nucleotide sequences [9]. This capability facilitates direct, real-time analysis of DNA or RNA fragments, drastically reducing sequencing time from days to mere hours.

Table 1: Selected NGS-Based Diagnostic Tests for Direct Pathogen Detection from Clinical Specimens

	Real Time PCR	**Sanger Sequencing**	**Targeted Next-Generation Sequencing (tNGS)**	**Metagenomic Next-Generation Sequencing (mNGS)**
Prior knowledge of the target	Yes	No	No	No
Enrichment of the target	N/A	Yes	Yes	No
Direct detection from clinical sample or microbial isolate required	Direct from sample or microbial isolate	Normally sterile sample or microbial isolate	Direct from sample or microbial isolate	Direct from sample or microbial isolate
Turnaround time	< 8h	< 8h	1-7 days	1-7 days
Relative ease of in-house implementation	Low	Low to Moderate	Moderate to High	High
Example of clinical application	Target-specific PCRs (i.e., *Mycoplasma pneumoniae,* methicillin-resistance in *Staphylococcus aureus*)	Microbial identification and strain typing (i.e., 16S rDNA sequencing)	Broad range PCR (i.e., universal fungal PCR)	Unbiased pathogen detection (i.e., Illumina UPIP, RPIP)

Practical Applications of NGS

Next-generation sequencing has several critical applications in clinical microbiology laboratories, including whole genome sequencing (WGS), metagenomic NGS (mNGS), and targeted NGS (tNGS). WGS involves sequencing and assembling the entire genome of a microorganism directly from a specimen or clinical isolate [10]. One of the primary uses of WGS is the simultaneous identification and typing of microbial pathogens for hospital and public health epidemiological studies. Compared to Sanger sequencing and traditional pulse field gel electrophoresis, WGS offers higher resolution and more comprehensive data. Furthermore, WGS is particularly valuable for detecting and characterizing resistance markers in clinical isolates, especially for gram-negative bacteria and members of the *Mycobacterium tuberculosis* complex [11]. Additionally, WGS is essential for identifying and characterizing emerging pathogens. For instance, in December 2019, lower respiratory specimens from pneumonia patients linked to a seafood market in Wuhan, China, were subjected to WGS. Bioinformatic analysis of these sequences identified an unknown pathogen matching the genome of lineage B betacorona

viruses, including SARS-CoV [12]. This newly identified virus was subsequently named SARS-CoV-2. Without the rapid capabilities of NGS-based WGS, culturing the virus for identification would have taken weeks to months. Thus, NGS-based WGS has revolutionized the rapid discovery of novel pathogens, significantly enhancing our ability to respond to outbreaks like the COVID-19 pandemic.

Metagenomic NGS (mNGS) enables the sequencing of all nucleic acids directly from patient specimens, including both pathogen and human DNA and RNA, without the need for culture [13]. This approach allows for unbiased detection of all microbial entities, resistance markers, and virulence factors, as well as host biomarkers linked to various disease states. This diagnostic method does not require prior knowledge of potential pathogens. Clinical assays have been developed to detect microbial nucleic acids from various specimen types, including blood, joint fluid, and cerebrospinal fluid (CSF), facilitating the diagnosis of diverse infections. However, a major limitation of mNGS is that host nucleic acids often dominate clinical samples, which can reduce the analytical sensitivity for detecting pathogens present at low abundance [14].

To address this, targeted NGS (tNGS) incorporates an enrichment process for microbial sequences of interest prior to library preparation, enhancing analytical sensitivity. The most common enrichment method for clinical applications involves the amplification of highly conserved regions of bacterial or fungal DNA before sequencing. For instance, in tNGS for bacterial detection, primers target and amplify the 16S ribosomal RNA gene, which is conserved across all bacteria [15]. Similarly, PCR-based enrichment techniques are employed to amplify SARS-CoV-2 RNA in clinical samples prior to NGS [16]. This process facilitates the detection of viral genome mutations, including those linked to resistance in viruses such as HIV, hepatitis B, and cytomegalovirus, directly from clinical specimens with high sensitivity. The enrichment step significantly boosts the number of target-specific sequencing reads by amplifying the nucleic acids of the target to millions of copies [17]. This contrasts with mNGS, where the majority of sequence reads originate from the host genome, thereby enhancing the sensitivity and accuracy of pathogen detection.

Clinical Applications of NGS Tests from Patient Samples

There is currently no prospective controlled clinical trial data assessing the effectiveness of NGS-based tests for unbiased pathogen detection directly from clinical specimens. The majority of existing literature consists of case reports or retrospective studies that compare the results of diagnostic NGS tests to those of standard care testing [18]. Theoretically, agnostic mNGS could provide a substantial advantage over traditional testing methods in certain patient populations, such as immunocompromised individuals who may be infected with rare or obscure pathogens, or in samples from patients who have previously received antimicrobial treatment, where culture-based tests might yield false-negative results.

Although numerous case reports highlight diagnoses achieved through mNGS that would have been missed with conventional testing, the overall clinical utility of mNGS remains uncertain when examined systematically. Several independent retrospective studies have reported limited effectiveness of mNGS assays, both in cerebrospinal fluid [19], joint fluid [20], and plasma [21], for unbiased pathogen detection. However, other studies have shown promise. For example, conventional diagnostic methods for the identification of *Mycobacterium tuberculosis* in cerebrospinal fluid, including culture, acid-fast bacillus stain, and PCR, have limitations such as low sensitivity and lengthy turnaround times. However, mNGS could serve as a frontline diagnostic tool for Tuberculous meningitis, offering higher sensitivity and faster turnaround times compared to traditional methods [22]. In joint fluid, mNGS identified pathogens in 94.9% of culture-positive prosthetic joint infection (PJI) cases, showing high concordance at the genus level (86.5%) and species level (73.0%) [23]. It also detected 15 pathogens in culture-negative cases and identified potential mixed infections, underscoring its utility in complex diagnostic scenarios. However, mNGS missed seven pathogens identified by culture, indicating areas for improvement. The findings suggest that mNGS could serve as a highly sensitive and specific diagnostic tool for PJI, particularly valuable in culture-negative cases and those with prior antibiotic exposure [23]. Integrating mNGS with conventional diagnostic methods could enhance pathogen detection and improve clinical outcomes for PJI patients. In plasma, studies demonstrate indicate that mNGS offers higher sensitivity and a broader pathogen detection range compared to traditional culture methods, making it a valuable tool for diagnosing acute intra-abdominal infections (IAIs) in sepsis patients, especially in culture-negative cases [24]. The rapid and comprehensive pathogen detection by mNGS can lead to more targeted and effective antibiotic therapy, reducing the use and duration of broad-spectrum antibiotics like carbapenems and anti-MRSA treatments. Overall, integrating mNGS with conventional diagnostic methods could significantly enhance the microbiological diagnosis and clinical management of sepsis and acute IAIs, leading to improved patient outcomes and optimized antibiotic use [24].

CONCLUSION

Overall, the application of NGS technology as a clinical diagnostic tool is still in its early stages. Despite its potential power, further research is needed to establish the optimal use and interpretation of results. Currently, NGS testing is confined to select reference laboratories, as the necessary instrumentation and technical expertise

are not widely available in most clinical labs. There is a need for optimization of NGS workflows to reduce cost and decentralize NGS testing platforms [7]. Additionally, although guidelines for method validation, result interpretation, and proficiency testing have been proposed, they have not yet been standardized across the field, hindering widespread adoption.

Advancements in NGS technology that continue to lower costs and the increasing availability of commercial bioinformatics tools will likely drive more widespread development of these tests. This progress could pave the way for future applications, including NGS diagnostics for a broader array of sample types. NGS possesses significant potential to transform the diagnostic landscape for infectious diseases. However, in its present state, NGS cannot supplant the existing standard of care testing. Moreover, current evidence does not advocate for the indiscriminate or screening-based use of NGS to rule in or out infections solely based on its results. The optimal application of NGS appears to be in cases where infection is suspected but conventional testing has yielded negative results. In such scenarios, it is imperative to consult with treating physicians, infectious disease specialists, and clinical microbiologists to ensure the appropriate utilization and interpretation of NGS test outcomes.

Lastly, molecular detection by mNGS does not provide antimicrobial susceptibility information, which may limit the ability to precisely target antimicrobial therapy and could inadvertently lead to prolonged use of broad-spectrum antimicrobials. These challenges underscore the necessity of conducting mNGS tests in collaboration with specialists in infectious diseases, clinical microbiology, and pathology to accurately interpret the clinical significance of the findings and their impact on patient management.

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