Metagenomic Next-Generation Sequencing (mNGS) for Pathogens Identification

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ABSTRACT

Metagenomic next-generation sequencing (mNGS) represents a transformative approach in identifying pathogens, significantly enhancing diagnostic capabilities in clinical microbiology. This technology allows for the comprehensive analysis of genetic material from diverse microbial communities without prior knowledge of specific pathogens. Using shotgun sequencing, mNGS can detect various infectious agent from a single clinical sample, including bacteria, viruses, fungi, and parasites. This unbiased sampling method is particularly valuable in diagnosing complex infections and tracking disease outbreaks, as it overcomes the limitations of traditional culture-based techniques. Furthermore, mNGS has the potential to predict antimicrobial resistance (AMR) by identifying resistance genes directly from clinical samples, thus aiding in public health surveillance and infection control strategies. Integrating advanced sequencing platforms and enrichment methods enhances the detection of low-abundance pathogens and resistance genes, providing a more accurate and timely response to emerging infectious diseases. Overall, mNGS is poised to revolutionize infectious disease diagnostics and surveillance, offering a comprehensive tool for monitoring and managing public health threats.

Keywords: metagenomic next-generation sequencing (mNGS); antimicrobial resistance (AMR); shotgun sequencing; infectious disease; surveillance; diagnostic

I. INTRODUCTION

Metagenomic next-generation sequencing (mNGS) is a powerful tool that combines the principles of metagenomics and next-generation sequencing (NGS) to analyse microbial and human genetic material in clinical samples. It can identify all types of pathogens, whether they can be grown in a lab or not, making it especially valuable for early and accurate diagnosis of infections. [1].

A. Overview of Metagenomic

Metagenomic, the study of genetic material recovered directly from environmental or clinical samples, has significantly transformed our understanding of human pathogens. This field has evolved over the past few decades, driven by advancements in sequencing technologies and bioinformatics, allowing researchers to explore the complexities of microbial communities associated with infectious diseases. [2]. Metagenomics platform initially focused on exploring microbial communities in various environmental settings, such as soil, oceans, wastewater and foods, better to understand their diversity, functions, and ecological roles. However, as the technology advanced, researchers quickly recognized its potential for clinical applications, particularly in infectious disease diagnostics. A major breakthrough for mNGS came in 2014, when metagenomic sequencing was first successfully used to diagnose an infection in a patient [3]. This milestone demonstrated the power of sequencing-based approaches to identify pathogens directly from patient samples, without the need for culture samples [4], [5]. It is an advanced and transformative scientific technique that allows researchers to explore and analyse microbial communities in their natural environments without the need first to grow them in a laboratory setting. By directly extracting and sequencing genetic material from diverse habitats such as soil, oceans, the human gut, and other ecosystems and this approach provides an unprecedented window into the hidden world of microorganisms [6].

Metagenomic provides a fascinating window into the hidden world of soil microbes, uncovering their incredible diversity and their vital roles in maintaining soil health. By analysing microbial DNA directly from the environment, researchers can identify countless unseen organisms and understand their contributions to nutrient cycling, plant growth, and disease prevention. This knowledge is transforming agriculture, helping farmers improve soil fertility, enhance crop yields, and develop more sustainable farming practices [7]. It reveals the hidden genetic potential of marine microorganisms that cannot be cultured in a lab, opening the door to groundbreaking discoveries. These tiny, elusive life forms harbour genes with immense economic and scientific value, from producing novel antibiotics to developing sustainable biofuels. By unlocking their secrets, researchers can tap into nature's vast biochemical library, paving the way for innovations that benefit medicine, industry, and environmental conservation [8]. By analysing the collective genetic material of microorganisms in a given environment, researchers can identify specific bacteria and fungi that contribute to the natural detoxification of contaminated sites. This knowledge not only deepens our understanding of microbial ecosystems but also paves the way for more effective and targeted bioremediation strategies, helping to restore polluted environments in a sustainable and efficient manner [9]. This technique is also widely used to monitor microbial communities in food products, which is crucial in ensuring both safety and quality. Carefully analysing the presence and balance of microorganisms helps detect potential contaminants, prevent spoilage, and maintain the desired characteristics of the food. This not only protects consumers from harmful pathogens but also ensures that products meet regulatory standards and maintain their expected taste, texture, and shelf life [10].

Through metagenomic, researchers can investigate the genetic makeup of entire microbial populations, gaining deeper insights into their taxanomy, evolutionary history, ecological roles, and overall diversity [6]. Beyond simply identifying which microbes are present, metagenomics helps assess microbial ecology by uncovering how different species interact, compete, or cooperate within a given environment. It also plays a vital role in disease surveillance and public health, offering a powerful tool for tracking emerging pathogens, monitoring antimicrobial resistance, and identifying potential outbreaks before they spread widely. Additionally, this technology is increasingly being applied in environmental and agricultural sciences, where it aids in monitoring soil health, detecting water contamination, and understanding the impact of climate change on microbial populations. By offering an in-depth and dynamic

perspective on microbial life, metagenomics continues to advance our understanding of the hidden but essential world of microorganisms [11].

B. Overview of NGS Technology

Next-generation sequencing (NGS) is a high-throughput sequencing technology that has revolutionized way researchers and clinicians analyse the genetic information of pathogens. This powerful technique allows for the rapid and comprehensive sequencing of entire genomes, transcriptomes (RNA), and epigenomes, providing unprecedented detail in genetic research. NGS has significantly accelerated discoveries in fields ranging from medical diagnostics to evolutionary biology by generating vast amounts of sequencing data in a relatively short time. NGS has revolutionized clinical diagnostics, making it easier and faster to detect genetic diseases, infectious pathogens, and cancer mutations more accurately. Its ability to sequence entire genomes or specific regions of interest has enhanced our understanding of disease mechanisms, allowing for more targeted and effective treatments [12]. One of the most powerful aspects of NGS is its ability to detect genetic variations such as mutations, insertions, deletions, and structural changes, which are the key to understanding inherited disorders, cancer, and infectious diseases. It has become an essential tool for precision medicine in clinical settings, helping doctors diagnose conditions more accurately and develop personalized treatments based on a patient's unique genetic profile. But its impact goes far beyond human health and NGS is also widely used in microbiology, agriculture, and environmental science, where it aids in studying microbial diversity, improving crop genetics, and monitoring ecosystems. By generating highly detailed genetic data on an unprecedented scale, NGS continues to drive major breakthroughs in medicine, genomics, and scientific research [13].

NGS has completely transformed genomic research, particularly in detecting and understanding pathogens. With its ability to rapidly sequence DNA and RNA at an unprecedented scale, NGS allows scientists to analyse vast amounts of genetic material in a fraction of the time it once took. This breakthrough has made it an essential tool for identifying pathogens, studying disease mechanisms, and advancing infectious disease research [14]. For instance, sequencing the human genome is a process that once took over a decade, with traditional Sanger sequencing, which can now be completed in just a day, making large-scale studies and personalized medicine more accessible than ever [15]. Additionally, by combining NGS with metagenomics (mNGS), researchers and clinicians can quickly and comprehensively detect microbes, diagnose infections, and explore microbial diversity in previously impossible ways. This innovative approach is helping to uncover hidden pathogens, improve disease diagnosis, and deepen our understanding of the microbial world that shapes both human health and the environment [16], [17].

C. Overview of mNGS workflows:

The mNGS workflow follows three key steps: sample preparation, sequencing, and bioinformatics analysis. A standard workflow consists of a series of steps that are generally divided into wet-lab and dry-lab processes [18]. The main objective is to extract, sequence, and analyse a sample's genetic material to better understand the microbial community present. This approach helps identify and characterize microorganisms, offering valuable insights into their diversity, composition, and potential role in health and disease [19].

i. Sample Preparation

The mNGS process starts with sample collection, a critical step in ensuring accurate results. Common sample types include blood, stool, cerebrospinal fluid (CSF), urine, and nasopharyngeal swabs, depending on the suspected infection [18], [20]. Samples like blood and CSF are often considered cleaner because they contain fewer background microbes, while others, such as stool or respiratory samples, may have a higher presence of commensal organisms, making analysis more complex [20]. To preserve the integrity of nucleic acids, especially for RNA viruses, it is essential to transport and process the samples as quickly as possible, minimizing degradation and ensuring reliable sequencing results [18]. The extraction of DNA and RNA from a sample is a crucial step in the mNGS workflow and is performed using specialized extraction kits. In cases where there is a strong suspicion of a specific pathogen, selecting an extraction kit that isolates only RNA or DNA can provide a more targeted and efficient approach. This is particularly important for samples with a high background of genetic material from other organisms, where the pathogen's nucleic acids may be present in very low quantities and risk being overshadowed. To ensure high-quality extraction, various commercially available kits can be used, such as RNeasy PowerSoil Total RNA Kit and RNeasy Mini Kit (Qiagen), while for viral samples, such as the MagMAX Viral Isolation Kit (Applied Biosystems) and Viral RNA Mini Kit (Qiagen), are commonly used. Choosing the appropriate extraction method helps maximize nucleic acid purity, yield, and integrity, ensuring more accurate sequencing results in downstream analyses [20].

The difference between using extraction kits and manual methods for isolating and purifying DNA and RNA in mNGS when each approach has its own advantages and limitations. Extraction kits from companies like QIAGEN provide standardized protocols that improve consistency, efficiency, and ease of use, significantly reducing processing time while ensuring high yield and purity of nucleic acids. Research suggests that automated extraction platforms often recover higher amounts of viral genomes compared to manual methods, which are more labor-intensive and can vary based on the skill of the operator [21], [22]. On the other hand, manual extraction methods can be more cost-effective and customizable, allowing researchers to adapt protocols to specific sample types or experimental needs. However, this flexibility comes with trade-offs, as manual techniques may result in lower nucleic acid yields and increased contamination risks, which could affect the overall quality and accuracy of mNGS results [23], [24]. DNA fragmentation is a crucial step in library preparation, and several methods are available. We compared nebulization, sonication, and enzymatic digestion to assess their impact on long-range PCR products in NGS. All three methods produced high-quality libraries for the 454 platform, but sequence coverage dropped significantly for fragments under 3,000 bp when pooling PCR products of different lengths. Overall, sequence quality and read length were comparable across methods. Enzymatic fragmentation was the most consistent but had slightly higher insertion/deletion rates.

However, after correcting for homopolymer errors, it aligned best with Sanger sequencing. Given the minor differences in performance, the choice of fragmentation method can depend on lab resources, feasibility, and experimental needs.[25].

Here is an example of library preparation for Oxford Nanopore MinION, which begins with DNA barcoding. This quick 15-minute step uniquely tags each sample. Next, the samples are pooled and cleaned up in a 25-minute process to ensure quality before moving on to adapter ligation, which takes just five minutes. Using compatible kits and flow cells throughout the protocol is crucial for accurate results. Finally, the prepared library is primed and loaded onto the flow cell, setting the stage for sequencing and downstream analysis with EPI2ME. The materials and equipment needed for this process include essential reagents like the Rapid Barcoding Kit, Monarch Genomic DNA Purification Kit, and Qubit assay kits, along with consumables such as MinION and GridION Flow Cells, PowerBead Pro tubes, and various buffers. It also covers additional reagents required to prepare a custom CTAB buffer and extract bacterial genomic DNA. Key tools like MinION/GridION devices, centrifuges, a Qubit fluorometer, and a range of pipettes are necessary on the equipment side. The section also specifies the required sample input for different extraction methods, whether using liquid culture or colonies, ensuring all steps from extraction to library preparation are well-supported with the right materials [26].

ii. Sequencing

Over the past 15 years, high-throughput DNA sequencing technology, commonly known as next-generation sequencing (NGS), has advanced incredibly. New and improved methods continue to emerge, making sequencing more accessible and efficient. As these technologies evolve, they open the door to an expanding range of applications, benefiting both fundamental research and real-world scientific advancements [27]. In mNGS, all nucleic acid fragments in the prepared library are sequenced. The choice of a sequencing platform depends on various factors, including the laboratory's research objectives, available resources, and user expertise. Among the available options, the Illumina MiSeq system is the most widely used for infectious disease research, pathogen surveillance, and discovery, particularly in public health settings. It is compact, user-friendly, and offers a relatively fast runtime, making it a popular choice for many laboratories. However, with ongoing improvements in sequencing technologies, platforms like the Oxford Nanopore MinION are emerging as valuable alternatives, especially for real-time surveillance and field applications in resource-limited settings. For example, the MinION was successfully used in the ZiBRA project for Zika virus surveillance in Brazil and in Guinea for real-time monitoring during the Ebola outbreak [28], [29]. In general, limited resources tend to use platforms such as Illumina (MiSeq and iSeq), Ion Torrent PGM, and MinION, while for substantial resources may opt for Illumina (NextSeq and NovaSeq), PacBio Sequel, or Oxford Nanopore PromethION, which offer higher throughput and greater sequencing depth. Additionally, to ensure quality and stability in pathogen sequencing, controls are essential. PhiX is commonly used as a sequencing control. In contrast, other essential controls include a positive control, an internal control (e.g., spiked DNA or a known pathogen), and a negative control, which typically is a water sample. These controls help validate the accuracy of the mNGS assay and maintain consistency over time [20]. Each platform serves a different purpose, such as:

a) **Illumina MiSeq**: Reliable choice for small-scale projects, delivering high-quality sequencing with a maximum read length of 2x300 bp. Each run generates up to 25 million reads, producing approximately 15 Gb of data. It is commonly used for targeted sequencing, small genome sequencing, and amplicon sequencing. Kraken2 software is used, as it works well with short-read sequencing [30].
b) **Illumina iSeq**: Compact and cost-effective sequencer with an easy-to-use workflow. It produces up to 1.2 million reads with a read length of 2x150 bp, making it ideal for smaller labs or low-throughput projects. It is suitable for smaller labs or projects requiring less throughput. Also use Kraken2 software is also used here, optimized for short-read sequencing and low-throughput data [31].
c) **Ion Torrent PGM**: Unique sequencing method that detects pH changes as nucleotides are incorporated into DNA strands. It supports read lengths of up to 400 bp and can generate between 15 to 60 million reads per run, depending on the chip used. It is ideal for targeted resequencing and small genome studies. Kraken2 software is used, as it handles Ion Torrent's error-prone homopolymer reads better than many other classifiers [32].
d) **Oxford Nanopore MinION**: Portable sequencer that uses nanopore technology to read DNA sequences. It can handle DNA fragments of any length and produce up to 20 Gb of data per run, offering real-time sequencing capabilities. It is useful for real-time sequencing in field settings and applications requiring long reads. EPI2ME software is used, which is optimized for long reads from Nanopore sequencing and enables real-time taxonomic classification [33].
e) **Illumina NextSeq**: Provides higher throughput than the MiSeq, generating up to 120 Gb of data and 400 million reads per run, with a maximum read length of 2x150 bp. It is suitable for whole-genome sequencing, exome sequencing, and RNA-Seq. Kraken2 software is used, which is optimized for short reads and can be paired with Bracken for improved species-level abundance estimation [34].
f) **Illumina NovaSeq**: One of Illumina's most powerful sequencing platforms, capable of producing between 167 Gb to 6 Tb per run, with read lengths of 2x150 bp. It can process many samples in a single run, making it a top choice for high-throughput sequencing. It is designed for large-scale genomic studies such as population genomics and large cohort studies. Kraken2 software is also used here, as it scales well with large NovaSeq datasets and can be paired with Bracken for accurate species-level abundance estimation [35].
g) **PacBio Sequel**: Utilizes Single-Molecule Real-Time (SMRT) sequencing, producing long reads (up to 40 kb) with high accuracy (99.9%) through circular consensus sequencing. Run times average around 30 hours.

It is effective for de novo genome assembly and resolving complex genomic regions. Centrifuge software is used, as it is optimized for long, high-error reads and handles complex metagenomic samples with accurate taxonomic classification [36].

h) **Oxford Nanopore PromethION**: An ultra-high-throughput platform that uses nanopore sequencing to analyse DNA in real time. It can produce up to 14 Tb of data per run, making it ideal for large-scale genomic projects. It is suitable for comprehensive genomic studies including structural variant detection. EPI2ME software is used for real-time taxonomic classification during sequencing, and it is optimized for long, high-error Nanopore reads [37].

For example, in sequencing and data analysis using the Oxford Nanopore MinION, ensure that you are using the latest version of MinKNOW for optimal performance, as updates improve basecalling accuracy. Open MinKNOW via the desktop shortcut, log in with Community credentials, and connect your sequencing device. After placing the light shield on the flow cell, set up your sequencing run by entering the experiment name, selecting the flow cell position, and choosing the FLO-MIN114 type. Next, select the appropriate Rapid Barcoding Kit (either the 24 or 96 version), keeping the default settings of a 72-hour run length and a 200 bp minimum read length. Make sure basecalling is enabled with the High Accuracy Basecaller (HAC) and that barcoding is turned on before proceeding. Configure output settings by selecting .POD5 for raw reads and .FASTQ is used for basecalled reads to ensure filtering is activated. Once sequencing begins, data processing and analysis can be performed using EPI2ME, where workflows like wf-bacterial-genomes help with bacterial and fungal genome assembly, species confirmation, MLST, and AMR prediction. Post-basecalling analysis is also carried out in EPI2ME, which supports Nextflow workflows. Finally, open the EPI2ME app using its desktop shortcut and navigate to the workflow tab to manage your analyses efficiently [26].

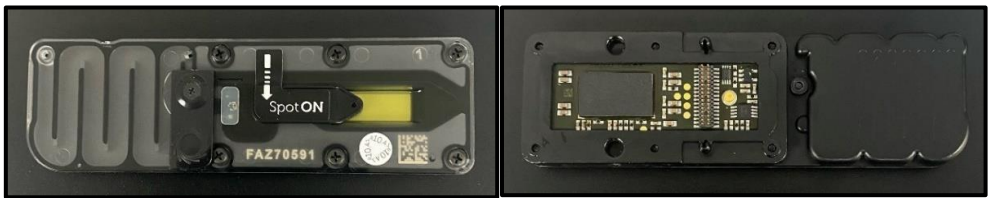

Figure 1: Example of MinION Flow Cell

iii. Bioinformatics

The Human Genome Project and other sequencing initiatives have generated an extraordinary amount of biological data, offering new insights into the blueprint of life. However, making sense of this vast information requires more than just raw data; it demands powerful analysis and interpretation tools. This is where bioinformatics comes in. As an evolving field, bioinformatics uses computational methods to organize, analyse, and extract meaning from biological data. It brings together expertise from computer science, mathematics, physics, and biology, creating a multidisciplinary approach that is transforming our understanding of genetics, medicine, and life itself [38].

The data analysis remains complex, requiring bioinformatics expertise and computational resources while sequencing has become more accessible. Raw sequencing data is cleaned, trimmed, and filtered to remove low-quality and duplicate reads. Host genome/transcriptome reads are eliminated to reduce background noise and improve pathogen detection. Further decontamination is done by comparing sample reads with a negative control to remove unwanted sequences. Large sequence reads are assembled *de novo* into contigs, which are identified by matching them to known genomes using BLAST. Tools like RAST and NCBI services are commonly used for gene annotation, with BEACON helping merge results for improved accuracy. Antibiotic resistance genes (ARGs) can be identified using CARD's Resistance Gene Identifier (RGI), while ShortBRED helps detect virulence factor genes using data from VFDB. These bioinformatics tools provide critical insights into microbial genetics, antimicrobial resistance, and pathogenic potential [20]. Figure 2 provides an overview of the essential steps involved in the NGS bioinformatics workflow, highlighting the processes carried out and the types of files generated at each stage [39], [40], [41].

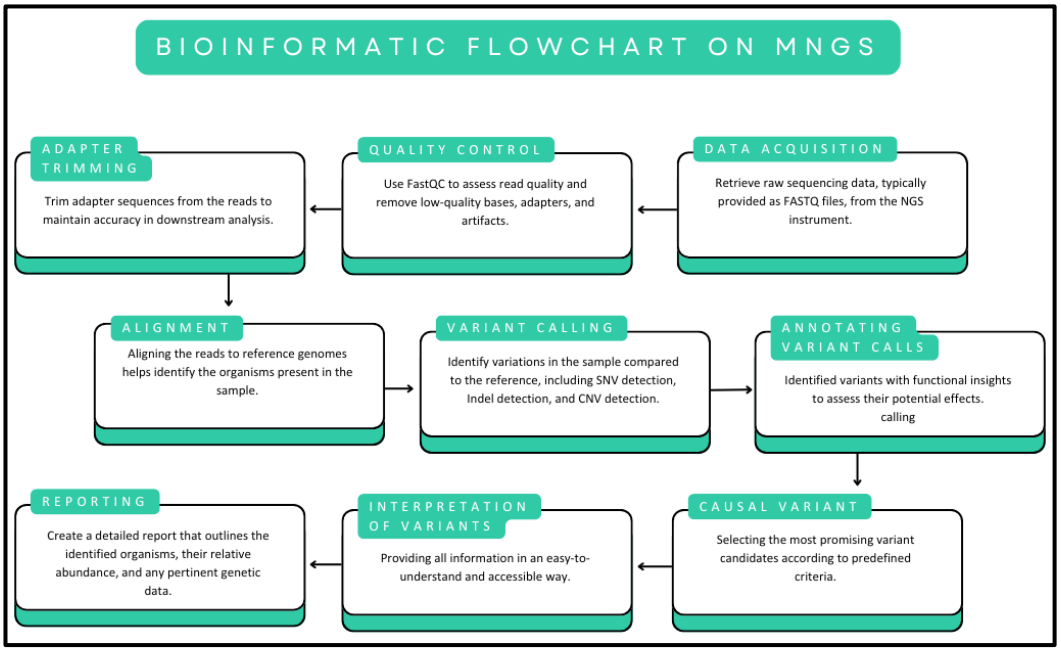

Figure 2: Key Stages of an NGS Bioinformatics Pipeline

D. Comparison with Traditional Methods

Traditional microbial identification methods have long been the cornerstone of clinical and environmental microbiology due to their reliability, affordability, and widespread availability [42]. These well-established techniques, such as culture-based methods and primer-based PCR, which it relies on end-point detection techniques, such as agarose gel electrophoresis, to visualize amplified DNA, have played a crucial role in detecting and identifying microorganisms [43]. However, despite their strengths, they have several limitations that can hinder comprehensive microbial analysis. This is where mNGS offers a transformative alternative by providing a more detailed, unbiased, and high-throughput approach to microbial identification. Traditional microbial identification methods depend on the growth and multiplication of microorganisms until they reach detectable levels. While these techniques have been widely used for decades, they are inherently slow and time-consuming. In many cases, it takes at least 48 to 72 hours to obtain results, and for certain slow-growing organisms, [42] such as *Mycobacterium tuberculosis* (MTB) and the process can stretch to several weeks before a definitive diagnosis is made. This delay can have serious consequences, particularly in severe or rapidly progressing infections. Patients may have to wait for a confirmed diagnosis before receiving appropriate treatment, leading to worsening conditions, prolonged hospital stays, and increased healthcare costs. In critical cases, such as sepsis or meningitis, where early intervention is crucial, waiting for traditional culture results can mean the difference between life and death. As a result, there is a growing need for faster, more efficient diagnostic techniques that can provide real-time pathogen identification. Advances in mNGS offer promising solutions by enabling rapid, unbiased detection of pathogens directly from patient samples, reducing diagnostic turnaround times, and improving patient outcomes [42].

Traditional microbial identification methods, while widely used and reliable in many cases, have certain limitations regarding sensitivity and specificity. These methods often rely on culturing microorganisms and analysing their biochemical characteristics, which can be challenging for certain species. Microbes with specific nutritional requirements or limited biochemical activity may fail to grow under standard laboratory conditions, leading to false negatives or incomplete identification [42]. Additionally, these techniques can struggle to differentiate closely related species, particularly those with similar morphological and biochemical traits [42]. This can result in misidentifications, affecting clinical decisions and treatment strategies. Some automated identification systems, while convenient, may also incorrectly classify certain species, further complicating the diagnostic process [44]. Given these challenges, newer technologies such as mNGS, offer a more advanced and accurate approach. By analysing the genetic material of all microorganisms present in a sample, mNGS can detect a broader range of pathogens, including those that are difficult to culture or distinguish using traditional methods, ultimately leading to more precise and timely diagnoses. Conventional testing methods often require labor-intensive, repetitive manual tasks that require significant time and expertise from skilled microbiologists. These processes typically involve culturing samples, preparing slides, staining, incubating, and performing biochemical tests, all of which must be meticulously carried out to ensure accuracy. Each step requires careful monitoring, precise handling, and thorough interpretation, making the workflow both time-consuming and resource-intensive [44].

II. ADVANTAGES OF mNGS IN PATHOGEN IDENTIFICATION AREAS

E. Improved Diagnostic Accuracy

In terms of accuracy, mNGS has significantly reduced misdiagnosis problems in a clinical setting through several mechanisms. mNGS utilised an untargeted method, which means all the sequences of all nucleic acids present in a sample will be identified, no matter what type of pathogens are present, whether they are bacteria, viruses, fungi, or even parasites. This comprehensive pathogen detection capability may solve the problems that the traditional method, especially its reliance on specific culture, may be unable to detect. For example, in cases where traditional methods yield negative results, mNGS can identify the pathogens while mNGS

can identify the pathogens, thus addressing the problem of misdiagnosis. A study found that mNGS could identify specific pathogens in children with severe pneumonia that traditional culture methods struggled to detect. These included *Bordetella pertussis*, *Pneumocystis jirovecii*, and cytomegalovirus, which typically require special growth conditions. The study also revealed that mNGS was far more effective at identifying mixed infections, detecting them in 48.9% of cases compared to just 4.3% with conventional methods [45]. Furthermore, mNGS is also known for its hypothesis-free diagnostic method that facilitates clinicians and researchers in simultaneously identifying a wide range of pathogens without any prior information on what to look for. This method is more likely to contradict the conventional method like targeted PCR, which mostly relies on specific primers for gene targets, to miss rare and unexpected pathogens. There is a study conducted in China, which stated that mNGS can detect a broader range of pathogens than ddPCR in bloodstream infection, where the study found that mNGS can detect 126 pathogens, while ddPCR that are well-known for its high sensitivity, is only able to detect 88 pathogens [46].

Moreover, the treatment outcomes also can be improved through this mNGS technique as this method is highly accurate and rapid in pathogen detection thus allowing for timely adjustments in clinical management, these elements are most highly important in severe infection where the option of antibiotic therapy can significantly affect patient survival and recovery. In patients with severe pneumonia, adjusting treatment from broad-spectrum antibiotics to targeted therapy based on mNGS results led to a significant drop in mortality rates, from 42.3% to 16.67% [47]. This remarkable improvement highlights how crucial accurate pathogen identification is in improving patient survival. Also, mNGS provides a significant advantage in diagnosing infectious diseases due to its high sensitivity and accuracy. This advanced approach can detect pathogens that traditional methods might overlook, leading to more precise diagnoses and better patient treatment and care. Sensitivity refers to how well a diagnostic test can accurately detect people who have a disease (true positive rate). Studies have shown that mNGS is far more sensitive than traditional diagnostic methods, making it a powerful tool for identifying infections more accurately. A meta-analysis found that mNGS had an impressive sensitivity of 97.4% (95% CI: 95.3–98.7) for diagnosing *Pneumocystis Jirovecii Pneumonia* (PJP), a serious opportunistic infection [48]. This means mNGS is highly effective at detecting PJP, even in cases where traditional tests might miss it due to low pathogen levels or unusual symptoms. While specificity refers to how well a test can accurately identify people who don't have a disease (true negative rate). High specificity is important because it helps avoid false positives and reduces the risk of unnecessary treatments. A systematic review of 3,205 clinical samples found that mNGS had 100% specificity for detecting Mycobacterium tuberculosis (MTB), ensuring all negative results were accurate [49]. This reliability is crucial in high-prevalence areas to prevent misdiagnosis and unnecessary treatments.

i. Enhanced Sensitivity and Specificity of Pathogen Detection

Regarding sensitivity in pathogen identification, mNGS is notably superior to the conventional traditional culture method. Studies have shown that mNGS has a high sensitivity, ranging from 88.3% to 92%, making it far more effective at detecting infections than traditional diagnostic methods. In contrast, conventional tests often have much lower sensitivity, typically ranging between 25% and 63%, depending on the type of test used [50], [51], [52]. This means that mNGS is far less likely to miss infections, allowing doctors to identify pathogens even in cases where traditional methods fail accurately. Higher sensitivity is crucial for detecting hard-to-culture or low-abundance pathogens, ensuring patients receive the right diagnosis and treatment sooner. For example, one study found that mNGS was 62.57% more effective than traditional methods in detecting pathogens, with a sensitivity of 88.3% compared to just 25.73% for conventional tests [51]. This highlights mNGS's superior accuracy, ensuring infections are identified more reliably and at an earlier stage, leading to faster and more effective treatments. Besides, mNGS is also more reliable pathogen detection than the conventional method and yields better and far more accurate results. In cases of cryptococcal meningitis, mNGS has shown a 92% sensitivity, making it far more effective than traditional culturing methods, which have a sensitivity of just 63.4%. This means mNGS can detect the infection much more reliably, ensuring patients receive quicker and more accurate diagnoses. Another conventional test, the CrAg test, has a slightly higher sensitivity at 96.7%, but mNGS still offers a significant advantage over standard culturing [50]. While CrAg detecting *Cryptococcus neoformans*, mNGS has the added benefit of identifying a wide range of pathogens in a single test, making it a powerful tool for diagnosing complex or co-existing infections.

Furthermore, the sensitivity of mNGS can vary depending on the type of clinical sample being tested, but it consistently outperforms traditional methods. For instance, in blood and cerebrospinal fluid (CSF) samples, mNGS has shown impressive sensitivities of around 90-91%, whereas conventional diagnostic techniques often struggle, with sensitivity dropping below 20-30% [53]. This difference is significant in detecting serious infections, such as bloodstream infections and central nervous system infections, where early and accurate diagnosis is very important for effective treatment. The ability of mNGS to identify pathogens quickly and reliably across different sample types makes it a powerful tool in various clinical settings, helping doctors make faster, more informed treatment decisions and ultimately improving patient outcomes. In summary, mNGS offers superior sensitivity in detecting pathogens compared to traditional diagnostic methods, regardless of the type of infection or clinical specimen. Its higher accuracy means infections can be identified earlier and more reliably, reducing the chances of missed diagnoses. This enhanced sensitivity is crucial in quicker and more precise diagnoses, allowing doctors to initiate targeted treatments without delay. As a result, patients receive the right therapy sooner, leading to faster recovery, fewer complications, and improved overall outcomes. With its ability to detect even hard-to-identify pathogens, mNGS transforms how infections are diagnosed and managed, making it an invaluable tool in modern medicine.

Table 1: Comparison between traditional method and mNGS in terms of sensitivity and specificity percentage.

Methods	Sensitivity	Specificity	Remarks	Source
Traditional (culture)	63.4% (26/41)	100% (20/20)	The values are given based on a positive result to all sample tests	[50]
	25.73%	88.41%	The values are given as the estimate, with the 95% CI in parentheses.	[51]

	(19.50–33.08%)	(77.89–94.51%)		
	57.8%	83.3%	The sensitivity value is based on comparison between mNGS and Culture with (P <0.01) while there were no differences in specificity between them (P > 0.05)	[52]
mNGS	92.0% (23/25)	100% (20/20)	The values are given based on a positive result to all sample tests	[50]
	88.30% (82.29–92.54%)	81.16% (69.57–89.21%)	The values are given as the estimate, with the 95% CI in parentheses.	[51]
	74.2%	83.3%	The sensitivity value is based on comparison between mNGS and Culture with (P <0.01) while there were no differences in specificity between them (P > 0.05)	[52]

ii. Comprehensive Pathogen Detection

mNGS is a specialized type of next-generation sequencing (NGS) that uses an untargeted approach, enabling comprehensive detection of all genes from all organisms present in a sample without requiring specific probes or primers for each potential pathogen. This powerful technique provides an unbiased approach to identifying bacteria, viruses, fungi, and parasites without needing prior knowledge of the potential pathogens [54]. Unlike traditional diagnostic methods, which often require multiple targeted tests to identify different pathogens, mNGS provides a broad, unbiased approach, making it especially valuable for detecting infections with unknown or unexpected causes. One of the key strengths of mNGS is the ability to accurately identify polymicrobial infections, which can detect multiple pathogens within the same sample. This is particularly important in cases where infections are caused by a mix of bacteria, fungi, or viruses, which traditional tests may miss or misidentify. By offering a more comprehensive and precise analysis, mNGS enhances diagnostic accuracy, supports timely and targeted treatment decisions, and ultimately improves patient outcomes in complex infectious disease cases [55].

mNGS is a powerful tool for detecting and identifying pathogens through a step-by-step workflow that ensures comprehensive microbial analysis. The process begins with sample collection, where clinical samples such as blood, cerebrospinal fluid, or bronchoalveolar lavage fluid are obtained from patients suspected of having infections. Next, nucleic acids (DNA and RNA) are extracted from these samples to prepare them for sequencing. Once extracted, the nucleic acids undergo library construction, where they are fragmented, and adaptors are added to enable sequencing. The prepared library is then processed through high-throughput sequencing platforms, such as Illumina, which utilizes sequencing by synthesis (SBS) to capture a broad range of microbial genomes, including bacteria, viruses, fungi, and parasites. After sequencing, advanced bioinformatics tools analyse the data, ensuring quality control, host sequence removal, taxonomic classification, and antimicrobial resistance gene detection. This allows for precise identification of pathogens, even in cases where traditional culture-based methods might fail, such as in complex infections or in patients who have received prior antibiotic treatment. By offering a detailed and unbiased view of the microbial community, mNGS significantly enhances the accuracy of infectious disease diagnosis and guides targeted treatment strategies [56].

iii. Rapid Diagnosis Time

Time is also an important element in diagnostic methods, and in other words, the quicker the diagnostic tool can yield, the more accurate and reliable the results, the better it is. One of the key advantages of mNGS is its faster turnaround time, typically delivering results within 96 hours from sample collection to reporting, depending on the laboratory's workflow. In contrast, traditional culture-based methods can take several days to weeks, especially for slow-growing organisms. For example, bacterial cultures often require three to five days for initial growth, with additional time needed for further identification and antibiotic sensitivity testing. This delay can be even longer in hard-to-culture pathogens, postponing critical treatment decisions. By providing rapid and comprehensive pathogen detection, mNGS enables doctors to initiate targeted treatments sooner, reducing hospital stays, minimizing complications, and ultimately improving patient outcomes. This speed is particularly crucial in severe or life-threatening infections, where every hour counts in guiding the right course of treatment. A study examining bronchoalveolar lavage fluid (BALF) samples found that mNGS delivered results in an average of 35.35 hours, making it significantly faster than traditional diagnostic methods [57]. In contrast, standard culture-based techniques often require several days to identify pathogens, delaying crucial treatment decisions. The ability of mNGS to detect infections within hours rather than days allows doctors to detect infections. In addition, a study comparing mNGS and traditional culture-based methods highlighted the significant difference in turnaround time (TAT) between the two techniques. Among 23 patients, mNGS provided results within one day, whereas only one out of 22 culture-based tests achieved the same speed. On average, mNGS delivered results in 1.19 days, while cultures took nearly four times longer at 3.86 days ($p < 0.001$), proving that mNGS is much faster and more efficient in identifying pathogens [58]. This rapid turnaround allows doctors to diagnose infections sooner, ensuring targeted treatments can be started without unnecessary delays. In summary, mNGS is revolutionizing infectious disease diagnostics, offering a faster, more accurate, and comprehensive alternative to traditional methods. Unlike conventional techniques, which can take days or even weeks, mNGS delivers results in a matter of hours to a few days. This speed and precision are crucial in critical cases, such as severe pneumonia, bloodstream infections, or meningitis, where delayed diagnosis can lead to complications or even be life-threatening. By enabling earlier and more targeted treatments, mNGS improves patient outcomes and helps reduce hospital stays, unnecessary antibiotic use, and overall healthcare costs. As technology advances, mNGS becomes an indispensable tool in modern medicine, paving the way for a new standard in infectious disease management that prioritizes efficiency, accuracy, and better patient care.

iv. Accelerated Patient Care

mNGS brings significant advantages to clinical diagnostics, especially regarding faster pathogen identification. Unlike traditional methods that can take days or weeks to grow and identify infectious agents, mNGS delivers results much more quickly, allowing doctors to make timely treatment decisions. This speed is especially critical in managing serious infections, where early and accurate diagnosis can mean the difference between rapid recovery and severe complications. By enabling quicker interventions and targeted treatments, mNGS not only improves patient outcomes but also helps reduce unnecessary antibiotic use, hospital stays, and overall healthcare costs. As a result, it is becoming an increasingly valuable tool in modern infectious disease management. This method can deliver results in an average of 35.35 hours, making it significantly faster than traditional diagnostic methods, which can take around 130 hours and, in some cases, up to 264 hours [59]. This drastic reduction in turnaround time means doctors can identify infections much sooner, allowing them to start targeted treatments without unnecessary delays. For patients with severe infections, every hour counts. Faster diagnosis means quicker interventions, better treatment outcomes, and a lower risk of complications. By enabling faster and more precise clinical decision-making, mNGS is transforming how infections are diagnosed and treated, ultimately improving both how infections are diagnosed and treated and improving patient care and hospital efficiency. Research has shown that mNGS can deliver results even faster in certain cases. For example, plasma mNGS testing has been reported to have a median turnaround time of just 28 hours, which is almost half the time required by conventional tests, which take around 57 hours on average [60]. Thanks to improved protocols, some laboratories can now deliver mNGS results in under 24 hours, especially for detecting viral respiratory infections [61]. This rapid turnaround is a game-changer, allowing doctors to identify the exact virus causing an illness and provide targeted treatment without unnecessary delays. For conditions like influenza, COVID-19, or other respiratory infections, early and accurate detection is crucial in preventing complications, guiding isolation measures, and reducing the spread of disease. As mNGS technology evolves, even more laboratories are expected to adopt these streamlined approaches, making fast, precise diagnostics the new standard in infectious disease management.

Furthermore, mNGS simplifies the diagnostic process by combining several critical steps, sample preprocessing, nucleic acid extraction, and bioinformatic analysis, into a single, streamlined workflow. This all-in-one approach makes the testing process more efficient, and timesaving compared to traditional methods, which often require multiple separate tests conducted in sequence, leading to longer processing times and potential delays in diagnosis [62]. By integrating these steps, mNGS not only speeds up the detection of pathogens but also reduces human error and resource usage, making it a more reliable and scalable diagnostic tool. This efficiency is particularly beneficial in cases where rapid and accurate results are essential, such as in critically ill patients or during outbreaks of infectious diseases. As a result, mNGS is helping to revolutionize clinical diagnostics by offering a faster, more precise, and more convenient alternative to traditional testing methods. With mNGS, doctors can identify infections much faster, allowing them to start targeted treatments right away. This speed is crucial for improving patient outcomes, as early intervention can prevent complications and improve recovery rates. For example, traditional culture-based methods can take 15 to 20 days to grow and identify certain pathogens, delaying treatment decisions [63]. In contrast, mNGS can provide results in just a fraction of that time, ensuring that patients receive the right medications sooner instead of relying on broad-spectrum antibiotics or waiting for confirmation. In real-world clinical settings, the speed and accuracy of mNGS have proven to be especially valuable in diagnosing complex and life-threatening infections, such as meningitis and encephalitis [64]. These conditions can progress rapidly, leading to severe complications or even death if not treated promptly. Traditional diagnostic methods often take days or even weeks to identify the exact pathogen, leaving doctors to rely on broad-spectrum treatments that may not be effective. mNGS, on the other hand, can detect the cause of infection in a matter of hours, allowing for faster, more precise treatment decisions. Integrating mNGS into clinical practice significantly speeds up pathogen detection, enabling more rapid diagnoses and timely treatments. This improvement leads to better patient care and outcomes. As technology advances, mNGS is poised to become a key tool in infectious disease management.

v. Culture-Free Approach

The culture-free approach of mNGS enhances how infectious diseases are diagnosed, offering significant advantages for clinicians and researchers. Unlike traditional culture-based methods, which require growing pathogens in the lab, a process that can be time-consuming, labour-intensive, and sometimes ineffective, while mNGS can bypass this step entirely. Instead, it directly analyses genetic material from patient samples, enabling the rapid and comprehensive detection of a wide range of pathogens, including bacteria, viruses, fungi, and parasites, even those that are difficult or impossible to culture. This means faster and more accurate clinician diagnoses, leading to earlier treatment. These targeted treatments can improve patient outcomes and reduce unnecessary antibiotic use. For researchers, mNGS provides valuable insights into emerging infectious diseases, antimicrobial resistance, and pathogen classification, helping to advance scientific understanding and public health efforts. mNGS has the unique ability to detect pathogens without requiring them to be alive or capable of growing in a lab, making it especially valuable in diagnosing infections caused by hard-to-culture organisms. For example, some fastidious pathogens, like Mycobacterium tuberculosis and Toxoplasma, are notoriously difficult to grow using traditional culture methods [65]. A study demonstrated that mNGS successfully identified these pathogens in samples that tested negative through conventional culturing, proving its effectiveness in detecting slow-growing, delicate, or challenging-to-culture organisms. Additionally, traditional culture-based diagnostic methods often have limitations, as they rely on specific growth conditions that may allow some pathogens to thrive while others go undetected. This selective nature can lead to missed diagnoses, especially in complex infections. In a study involving patients with joint infections, mNGS has been able to identify eleven pathogens that traditional cultures failed to detect, including *Staphylococcus lugdunensis* and *Coxiella burnetii* [66].This highlights mNGS's unique advantage: It provides unbiased and comprehensive pathogen detection by analysing all genetic material in a sample rather than relying on an organism's ability to grow in a lab. By overcoming the growth limitations of traditional methods, mNGS ensures that no potential pathogen is overlooked, leading to more accurate diagnoses and improved patient care.

F. Guiding Antibiotic Therapy

mNGS is a powerful tool for guiding antibiotic therapy, offering accurate pathogen identification and enabling timely treatment adjustments. Research has shown that mNGS improves infection diagnosis, especially in complex cases like severe central nervous system (CNS) infections and febrile neutropenia (FN). Compared to traditional methods, mNGS has a higher detection rate, identifying mixed infections, previously undetected pathogens, and even fungal infections that might otherwise be missed. In clinical practice, mNGS results help optimize antibiotic therapy by allowing healthcare providers to add, adjust, or de-escalate treatments based on the specific pathogens identified. This targeted approach not only improves treatment effectiveness but also helps reduce unnecessary antibiotic use, lowering the risk of antibiotic resistance. Overall, mNGS is transforming infectious disease management, enabling more precise, personalized, and effective antibiotic treatments to improve patient outcomes [67], [68], [69]. It is also highly effective in identifying rare, novel, and hard-to-detect pathogens, including cases of coinfection, directly from clinical samples. It also holds great promise for antibiotic resistance prediction by sequencing resistance genes, providing valuable diagnostic insights to help guide treatment decisions and improve antibiotic stewardship. While many physicians view mNGS as a last-resort method for challenging infection cases, its potential is undeniable. However, challenges such as workflow validation, quality control, standardization, and data interpretation still need to be addressed before mNGS can be routinely implemented in clinical laboratories [70].

Fortunately, these hurdles are temporary and can be overcome with rapid technological advancements. As workflows become more validated, costs and turnaround times decrease, and data interpretation becomes more streamlined, mNGS is expected to become a widely accepted tool in clinical diagnostics [70]. mNGS surpasses conventional methods in detecting unknown and mixed infections, improving pathogen identification in lung infections. This is crucial for guiding antibiotic therapy, reducing reliance on broad-spectrum antibiotics, and preventing antibiotic resistance. By providing faster, more precise diagnoses, mNGS helps clinicians optimize treatment, ensuring targeted therapy while minimizing unnecessary antibiotic use. Rather than replacing conventional methods, mNGS complements them, enhancing early, personalized treatment and supporting antibiotic stewardship to combat antimicrobial resistance [71].

G. Novel Pathogens Discovery

mNGS is a versatile and powerful tool that is crucial in infectious disease diagnostics and microbial research. It is particularly valuable for identifying unknown pathogens, allowing clinicians and researchers to detect contagious agents without prior knowledge of their genetic sequences. Additionally, mNGS provides detailed insights into complex microbial communities, enabling the study of microbial diversity, interactions, and ecological roles within a given sample. Beyond pathogen identification, mNGS is also used to detect infectious diseases and screen drug resistance genes, helping to guide more effective treatment strategies. Thanks to its high-throughput sequencing capabilities, mNGS can be applied in clinical settings to uncover novel pathogens or investigate complicated infections where traditional diagnostic methods fall short. Its ability to provide a comprehensive and unbiased analysis makes it a game-changing technology in the fight against infectious diseases, improving patient outcomes and advancing precision medicine [72]. The assay utilizes *de novo* assembly and translated nucleotide algorithms, allowing it to identify novel and sequence-divergent human viruses by recognizing similarities to related animal or plant viruses. This capability is particularly valuable in detecting previously unknown or emerging pathogens, as it does not rely on prior knowledge of their genetic sequences. Unlike traditional diagnostic methods, which often require specific primers or probes, mNGS can uncover unexpected or genetically distant viruses that might otherwise go undetected. By analysing the full spectrum of genetic material in a sample, mNGS enables researchers and clinicians to track viral evolution, monitor cross-species transmission, and enhance epidemic preparedness. This makes it an indispensable tool in public health surveillance, helping to identify new viral threats before they become widespread. As sequencing technology continues to improve, the ability of mNGS to detect and characterize emerging pathogens will play an increasingly critical role in global infectious disease management [67].

Due to its extensive sequencing capabilities, mNGS has become a powerful tool in clinical diagnostics, particularly for identifying novel pathogens and uncovering complex sources of infection. Traditional diagnostic methods, such as culture-based techniques or targeted molecular tests like PCR, require prior knowledge of the suspected pathogen, limiting their effectiveness when dealing with unknown or unexpected infections. In contrast, mNGS provides an unbiased, broad-spectrum approach, allowing to detect bacteria, viruses, fungi, and parasites in a single test without the need for specific primers or probes. This makes mNGS especially valuable in cases of mystery infections, where standard tests fail to yield a diagnosis, or in critically ill patients, where rapid and accurate pathogen identification is crucial for timely treatment. Additionally, mNGS can detect co-infections or antimicrobial resistance genes, providing clinicians with a more comprehensive understanding of the infection and guiding more effective treatment strategies. By offering more profound insights into the microbial composition of a sample, mNGS is transforming infectious disease diagnostics and paving the way for more personalized and precise medical care [73]. mNGS an unparalleled advantage in rapidly identifying novel pathogens due to its ability to detect a broad range of microorganisms without prior knowledge of the specific culprit. This capability was crucial during the early stages of the COVID-19 outbreak. For example, in 2019, clinical data and bronchoalveolar lavage fluid samples were collected from five patients with severe pneumonia at Jin Yin-tan Hospital [74]. By extracting and sequencing the nucleic acids using the mNGS approach, they identified a previously unknown beta-coronavirus strain belonging to a distinct clade and sharing 79% nucleotide identity with SARS-CoV. Beyond pathogen discovery, mNGS is vital in tracking infection sources, predicting transmission patterns, and analysing genetic mutations. These capabilities make it an invaluable tool for understanding emerging infectious diseases and improving public health responses [75].

H. Cost-Effectiveness in Long Term

mNGS significantly improves pathogen detection compared to traditional methods, leading to more accurate diagnoses and better-targeted treatments. By identifying the exact cause of an infection, doctors can prescribe the most effective medications, reducing the need for broad-spectrum antibiotics and unnecessary treatments. A study found that when mNGS was used alongside

conventional diagnostic methods, it helped save an average of 3,069 CNY (about 453 USD) per patient [76]. These savings came from more effective treatment plans, reduced hospital stays, and lower drug costs, highlighting precision medicine's financial and clinical benefits. By providing faster and more accurate diagnoses, mNGS can help reduce the length of hospital stays. For example, research has shown that pneumonia patients diagnosed using mNGS tended to be discharged sooner than those diagnosed with traditional methods [77]. Next, shorter hospital stays mean lower medical expenses for patients and their families and reduced reliance on costly treatments and extended care. Additionally, this efficiency eases the strain on healthcare facilities, freeing up hospital beds and resources for other patients in need. By improving patient outcomes and hospital operations, mNGS plays a valuable role in making healthcare more effective and sustainable. Although mNGS may have higher upfront costs than conventional diagnostic methods, its long-term benefits can make it a cost-effective investment. By enabling faster and more precise diagnoses, mNGS helps reduce hospital stays, minimizing patient and healthcare facility expenses. It also decreases the reliance on broad-spectrum antibiotics, lowering the risk of antibiotic resistance and unnecessary drug costs. In short, while the upfront cost of mNGS may be high, its ability to provide more accurate diagnoses, shorten hospital stays, and lower readmission rates makes it a worthwhile investment in the long run. Improving patient outcomes and optimizing treatment plans, mNGS helps reduce healthcare costs. As more hospitals and clinics integrate this technology, its benefits will likely become even more evident, making it an essential tool for modern healthcare. According to figure 2, improved patient outcomes mean fewer complications, readmissions, and prolonged treatments, all contributing to overall healthcare savings. As mNGS becomes more widely adopted in clinical settings, these financial advantages will likely outweigh the initial investment, making it a valuable tool for cost efficiency and better patient care[78]

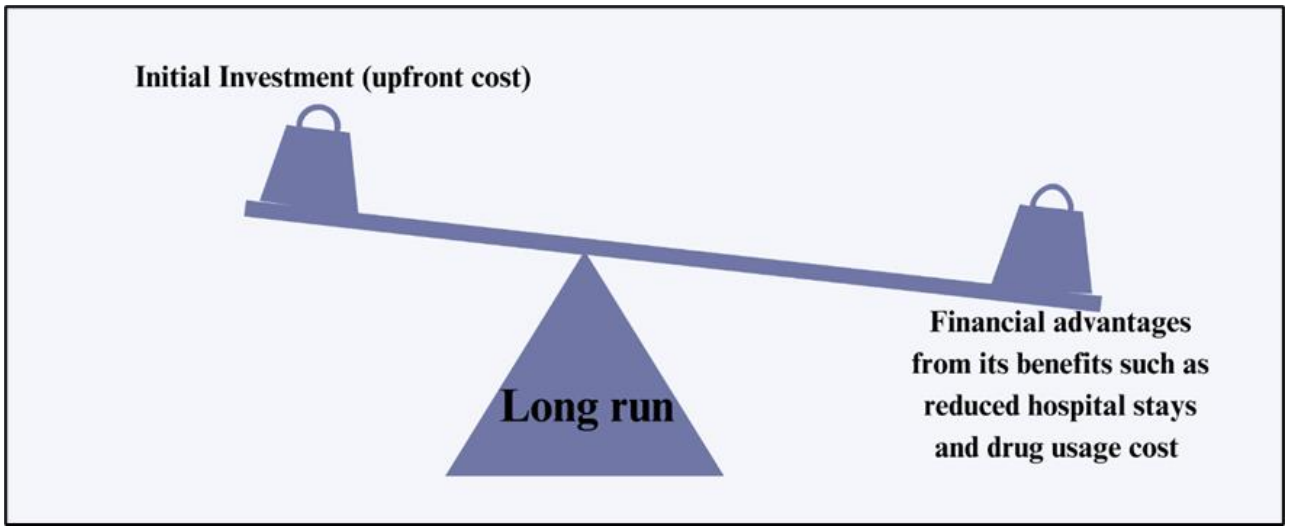

Figure 2: Illustration to represent how mNGS economically benefits the healthcare sector in the long run.

III. LIMITATIONS

The application of mNGS in pathogen identification, although promising, is also faced with several limitations that hinder its broad clinical implementation. These limitations include:

I. Sample Quality Issues

The quality of the sample highly correlates with the accuracy and reliability of the results that correlate with the accuracy and reliability of the results obtained from mNGS. Poor sample quality may be caused by contamination, degradation, or inadequate microbial content, which can lead to introducing some errors, cover up important findings, and lead to incorrect conclusions. In clinical samples, the overwhelming presence of human DNA or RNA is known as host background interference, which may conceal microbial pathogens. A significant amount of host-derived nucleic acids is present in the sample due to the human genome's size, which is significantly larger than that of microorganisms. Over 99% of the sequencing data can be made up of human DNA and RNA, leaving only a tiny fraction for microbial genetic material [79]. This is like trying to find a few grains of sand on a vast beach as the microbial signals are easily overwhelmed by the sheer volume of host genetic material. As a result, detecting pathogens or analysing the microbiome becomes much more challenging. This high background noise significantly reduces the sensitivity of mNGS to detect pathogens. To address this issue, researchers employ specialised methods to lessen the overwhelming amount of human DNA in clinical samples, which facilitates the detection of microorganisms. One strategy is hosting DNA depletion, in which techniques are utilised to remove human genetic material in a targeted manner before sequencing, thereby preserving a more significant percentage of microbial DNA for analysis. Targeted sequencing is another approach that lets researchers concentrate on the microbial community without getting overwhelmed by human DNA by concentrating only on particular microbial markers, like the 16S rRNA gene for bacteria [80]. These methods contribute to metagenomic sequencing's increased sensitivity, which enhances its usefulness for microbiome research and infection diagnosis.

Due to their high sensitivity, metagenomic samples are easily able to detect undesirable microorganisms from the environment, laboratory apparatus, or even the chemicals (reagents) used in processing [81]. Think about attempting to hear a particular voice in a crowded space; background noise can make it challenging to identify the correct individual. In the same way, contamination adds additional "noise" to sequencing data, making it difficult to discern between actual pathogens and unintentional invaders. False-positive results may result, in which unimportant bacteria or laboratory pollutants are mistakenly classified as possible dangers. To guarantee that the final data appropriately reflects the sample's microbial composition, researchers must handle samples with extreme caution, adhering to stringent protocols and contamination controls. In metagenomic sequencing, stringent quality control procedures

are essential to guaranteeing accurate results. Using negative controls is a significant step [79]. These are the samples that shouldn't contain any microbial DNA; if something still shows up in the sequencing data, it's probably contaminated. The chemicals used to process samples, known as reagents, are also tested before use to ensure that no undesired microbial traces are being introduced. Additionally, routine environmental testing aids in identifying any possible sources of lab contamination, such as bacteria that remain on equipment or airborne microbes.

J. Data Analysis Complexity

The mNGS generates millions of short DNA sequences from the sample, which results in a massive amount of data. Imagine attempting to put together a vast jigsaw puzzle made up of millions of tiny pieces, many of which have similar appearances. It is challenging to analyse and interpret due to the enormous amount of data. Specialised bioinformatics tools that can process, sort, and identify these DNA sequences are used by researchers to help them make connections between them. It isn't easy to manage such vast amounts of data effectively, though. Powerful computers and sophisticated algorithms are needed to synthesize significant insights because current software may not handle the massive computational demand [82]. To accurately identify microbial DNA while removing noise and contamination, speed is an important aspect to be considered. Metagenomic sequencing breaks down DNA into millions of tiny pieces called reads. This makes High throughput sequencing possible, but it also poses a significant challenge because it is difficult to assemble these tiny fragments to reconstruct entire genomes. Due to the short reads, the assembly process frequently produces fragmented genomes, so researchers are left with fragments of genetic information rather than a complete microbial genome [83]. Therefore, it is more difficult to understand the significance and evolutionary connections of the microorganisms in a sample fully. Since this technology contains genetic material from various microorganisms, such as bacteria, viruses, fungi, and other microbes, metagenomic samples are incredibly complex [84]. In contrast to conventional DNA sequencing, which concentrates on a single organism, metagenomics records every detail in a sample, resulting in a mixture of DNA from various species. Because of this diversity, there is a huge data challenge. To determine which microbes are present and what functions they serve, the innumerable short DNA fragments (reads) contributed by each organism must be sorted, put together, and examined. Accurately connecting the sequences, the microbial population becomes more challenging and more diverse. Although scientists classify these sequences using sophisticated databases and algorithms, figuring out metagenomic data is still difficult and resource-intensive, despite the latest technological advancements.

Data analysis also brings to another problem to the clinical applications setting. Analysing mNGS data can be challenging, particularly in clinical settings where prompt and precise results can affect patient care. Simply running a sample through a sequencer is insufficient due to the volume and complexity of the data; experts must carefully interpret the results to distinguish between background noise and actual infections. For this reason, a multidisciplinary team is important to overcome this problem by working together to develop rapid and accurate diagnosis and bring proper treatment to the patients [85]. Infectious disease specialists offer the medical background necessary to ascertain whether a pathogen that has been identified is causing a patient's symptoms, computational biologists create and apply algorithms to process enormous volumes of sequencing data, and microbiologists assist in identifying and comprehending the present microbes. In the absence of this multidisciplinary cooperation, there is a chance that misinterpretations could result in missed diagnoses or needless treatments. By integrating knowledge from several disciplines, hospitals and research labs can guarantee that mNGS results are precise, dependable, and genuinely helpful for patient care. Thus, a number of advancements are required before mNGS can reach its full potential in clinical settings. A significant obstacle is data analysis's intricacy, necessitating more sophisticated bioinformatics tools that can efficiently and precisely handle enormous volumes of sequencing data. Genetic material's sheer amount and diversity frequently overwhelms current software, so creating more effective algorithms and intuitive platforms is crucial. These developments bring mNGS one step closer to becoming a standard and very useful instrument for microbiome research and infection diagnosis.

K. Resource Limitations

When talking about resource use in mNGS, most likely correlates with the cost that is needed, even though mNGS is a cost-saving method in the clinical setting, especially in the long run, as it gives more benefits towards the patient’s outcome, which will later cut some costs, such as medications and hospital care. However, to initiate this technology, especially in a hospital, is not cheap as using the traditional method like culturing one. This high initial cost of mNGS is one of the primary difficulties in implementation and may prevent widespread deployment in many hospitals. For just five samples, the reagents necessary for the sequencing process alone cost about SGD 2,800 [86]. This is only the beginning, though. Several additional costs in addition to the cost of the reagents increase the price. Each run requires the use of laboratory supplies like pipette tips, sequencing plates, and sample preparation kits. The financial burden is further increased by overhead expenses such as expert staff salaries, bioinformatics processing, and equipment maintenance. The overall cost also includes administrative fees for processing and reporting results. On the other hand, mNGS technology setup involves more than just purchasing the equipment; it involves other substantial expenses that may be prohibitively expensive for labs with limited resources or smaller operations. Labs require more equipment than just the sequencer, such as powerful computers, storage spaces, and hygienic workspaces. Staff training is also essential given the specialised skills needed to operate and interpret NGS data. Furthermore, continuing maintenance raises overall costs, including software upgrades, instrument calibration, and consumable replacements. To make NGS more accessible for smaller labs, funding support, shared facilities, or more reasonably priced equipment options are required. These costs may be manageable for well-funded institutions.

Large volumes of data are produced by mNGS, which requires proficient bioinformatics tools for efficient processing and interpretation. This includes strong computing facilities to manage the massive amount of data, specialised software to evaluate and filter sequencing data, and skilled bioinformatics specialists for ensuring accurate results. However, a lack of trained staff and limited access to high-performance computing causes many institutions to struggle with these demands [87]. Analysis of mNGS data may become slow, ineffective, or unreliable without adequate resources. These obstacles can be addressed by increasing access to cloud-based computing, bioinformatics training programs, and user-friendly software, making mNGS more widely available in various labs

and healthcare environments. Despite the difficulties, mNGS is becoming more widely available due to technological advancements and falling costs. Low- and middle-income nations can more easily implement this technology without needing costly infrastructure thanks to innovations like Oxford Nanopore Technologies, which provide portable, reasonably priced sequencing solutions [87]. Meanwhile, advancements in reference databases and bioinformatics tools make data analysis easier and enable more precise microbial identification. Standardizing processes from sample preparation to data reporting helps guarantee consistent and trustworthy outcomes across various labs. With further development, mNGS could emerge as a potent and popular clinical diagnostic tool that improves how we identify and investigate infectious diseases, even in environments with limited resources.

IV. APPLICATIONS OF mNGS IN PATHOGEN IDENTIFICATION

By providing a quick, extremely sensitive method of identifying infectious agents in clinical samples, mNGS has completely changed how everyone identifies and investigates pathogens. mNGS can simultaneously identify bacteria, viruses, fungi, and parasites from a simple sample, in contrast to conventional diagnostic techniques that frequently test based on clinicians' presumption of the predicted pathogen. Because of this, it is a handy tool for diagnosing infectious diseases, particularly in complicated cases where conventional testing cannot identify the cause. In addition to healthcare, mNGS is essential for food safety since it helps detect dangerous microorganisms in food items before they are consumed. It is also frequently employed in biosurveillance, enabling researchers to monitor emerging infectious diseases and track antibiotic resistance globally.

L. Infectious Disease Diagnosis

The diagnosis of infectious diseases is being improved by mNGS, which provides a more rapid, thorough, and highly sensitive method than conventional techniques. By examining all the genetic material in a sample at once, mNGS utilises an unbiased strategy in contrast to traditional testing, which frequently requires physicians to suspect a particular pathogen before conducting targeted tests. This implies that multiple tests are not necessary because it can simultaneously detect bacteria, viruses, fungi, and parasites, even uncommon or unexpected ones. Because mNGS analyses all the genetic material in the sample, it takes a broad, objective approach that makes it particularly useful in unclear or complex cases. This capability is especially lifesaving for immunocompromised patients, such as those receiving chemotherapy or organ transplants, where unusual or unexpected microbes may bring on infections [88]. Additionally, it is very helpful in identifying novel, rare, or emerging pathogens that conventional diagnostic testing might miss. mNGS assists physicians in making quicker, more precise treatment decisions by eliminating the need for numerous, focused tests and offering a thorough, data-driven diagnosis, ultimately leading to better patient care and results. A study that compared mNGS with conventional diagnostic techniques (TDMs) found that mNGS had akin specificity (83.3% vs. 89.6%) but a higher sensitivity (59.7% vs. 30.1%). Bacteria (88.7%), fungi (87.9%), viruses (96.9%), and nontuberculous mycobacteria (NTM) (100%) all had noticeably more excellent detection rates with mNGS [89]. Based on these statistics, it can be concluded that mNGS technology is far superior to conventional methods and should be implemented in all clinical setting worldwide as infectious diseases have become even more complicated and hard to detect.

The fact that some pathogens are very difficult, or even impossible to grow in a lab using conventional culture techniques is one of the main problems with traditional diagnostics. Many bacteria, known as the fastidious bacteria, are challenging to identify using traditional techniques because they need suitable conditions to survive. In the same way, some viruses are difficult to culture, which results in missed diagnoses. By directly examining the genetic material in a sample without first growing the pathogen, mNGS gets around this restriction. This makes it possible to swiftly and precisely identify even difficult pathogens, those that require weeks to cultivate or do not grow at all [90]. mNGS offers a potent tool for diagnosing infections that might otherwise go unnoticed by identifying these difficult-to-culture pathogens. Additionally, mNGS is essential for quick and precise infection diagnosis, which is particularly critical in critical care environments such as intensive care units (ICUs) [91]. Doctors can make quicker, more targeted treatment decisions by using mNGS to identify the precise pathogen causing the infection and identify the precise pathogen causing the infection and its genomic features, such as genes that resist antibiotics. mNGS enables precise therapy, guaranteeing the appropriate medications are used depending on trial-and-error antibiotic use, which can be time-consuming and worsen a patient's condition. In high-risk hospital settings, this enhances patient outcomes and aids in infection control by preventing the spread of bacteria resistant to drugs. The ability of mNGS to offer quick and thorough diagnostic insights can be lifesaving for critically ill patients, where every minute matters. According to a study conducted in China, mNGS has demonstrated significantly higher sensitivity in detecting central nervous system (CNS) infections than traditional diagnostic methods. In the survey, mNGS identified 17 pathogenic viruses, more than double the number detected by conventional methods, with a positive detection rate of 66.28% for CNS viral infections. Similarly, mNGS detected 13 pathogenic bacteria for bacterial infections, compared to just five identified by standard techniques, achieving a detection rate of 65.71%. When it came to fungal infections, mNGS also outperformed traditional methods, identifying five pathogenic fungi, while conventional diagnostics found only 3, with a detection rate of 62.96% [92]. These findings highlight the power of mNGS in uncovering a wider range of pathogens, including those that might be missed by culture-based or targeted tests. By providing a faster, more comprehensive approach to diagnosing CNS infections, mNGS enables earlier, more precise treatment, ultimately improving patient outcomes especially in critical cases where rapid intervention is essential. When every challenge is well addressed, mNGS offers thorough pathogen detection capabilities that can enhance patient outcomes and direct focused treatments, marking a substantial advancement in infectious disease diagnostics.

M. Tickborne and Parasite Pathogens Detections

In clinical settings, mNGS is a handy tool for identifying parasitic infections and tickborne illnesses, providing several benefits over conventional diagnostic techniques. When diagnosing complex or co-infections in which multiple pathogens are involved, this is very useful. Furthermore, mNGS is an essential tool in situations where conventional tests are unable to yield answers because it can detect novel or unexpected pathogens, especially in parasites, as the parasites and ticks can transmit and carry various kinds of pathogens, which are complex to detect by the traditional culture method fully. Thus, mNGS assists physicians in making more informed

treatment decisions by providing a swift, thorough, and objective method of diagnosing parasitic and tickborne infections, ultimately improving patient care and results. This is especially crucial for diseases carried by ticks, which can be challenging to identify because of their diverse symptom sets and overlapping clinical manifestations. Waiting for conventional test results and getting inconclusive results can delay life-saving interventions because early treatment is crucial to preventing serious complications. By rapidly and precisely identifying the infectious agent, mNGS helps doctors overcome these obstacles and begin focused treatment earlier. In addition to improving patient outcomes, this lowers the chance of long-term issues like severe rickettsial infections or untreated Lyme disease. mNGS guarantees that patients receive the appropriate treatment promptly by providing a quick, objective, and thorough diagnosis approach, eventually improving recovery and healthcare results. One study demonstrated how well mNGS can identify the bacteria Rickettsia and Coxiella burnetii, which cause serious tickborne illnesses like Q fever and rickettsioses. According to the results, mNGS successfully identified these pathogens, and PCR tests confirmed infections in 84.6% of patients [93]. All patients experienced notable improvements because the doctors' quick and precise diagnosis enabled them to prescribe targeted treatments. These infections may not have been identified or misdiagnosed as other illnesses in the absence of mNGS, which would have delayed appropriate treatment and raised the possibility of complications. mNGS is an indispensable tool in managing infectious diseases by facilitating early and accurate pathogen detection. This helps physicians make quicker, better-informed treatment decisions, improving patient outcomes. In another study, mNGS showed a positivity rate of 53%, successfully identifying various microorganisms, including parasites, that might go undetected with conventional tests [94]. This means that mNGS is unambiguously proven to be utilised in hospital settings, especially in areas where they are highly exposed to parasites and ticks, as it will facilitate the clinicians' diagnosis there, thus providing better treatment for the patients. A study in Inner Mongolia showed how effective mNGS is at identifying tickborne diseases in the area. By analysing pooled tick samples using mNGS, researchers identified many significant disease-causing microbes, such as Rickettsia, Anaplasma, and Coxiella, which are pathogens known to cause serious illnesses in humans and animals. Individual tick DNA samples were put through genus-specific PCR testing to validate these results further, and the results verified the presence of these pathogens [95]. This study shows how well mNGS works for extensive tick surveillance, assisting researchers and public health officials in monitoring the spread of diseases carried by ticks, spotting new dangers, and creating more effective preventative strategies to protect the health of people and animals. In conclusion, mNGS is an effective method for identifying parasite and tickborne infections due to its high sensitivity and wide pathogen coverage. Its quick pathogen identification and clinical diagnosis modification capabilities highlight its usefulness in clinical settings. More research is necessary to measure its efficacy in identifying parasite pathogens completely.

N. Food Safety Control

mNGS improves food safety by thoroughly understanding the microbial communities in food products and production environments. mNGS can simultaneously identify a large variety of microbes in a single test, in contrast to traditional testing methods that frequently concentrate on detecting particular bacteria or pathogens. Because it enables them to identify possible sources of foodborne outbreaks, detect contamination early, and guarantee higher safety standards, it is a vital tool for public health officials, regulatory bodies, and food manufacturers. mNGS increases consumer confidence in food safety procedures, lowers product recalls, and prevents foodborne illnesses by offering quick and precise insights into the microbiological nature of food.

i. Early Detection of Pathogens

mNGS can quickly and concurrently identify foodborne pathogens from a single test, such as *Salmonella typhi, Escherichia. coli, and Listeria monocytogenes*, *Campylobacter jejuni*, *Vibrio vulnificus*, and *Cronobacter sakazakii.* This objective method increases detection speed and improves accuracy by spotting even minute amounts of contamination that conventional testing might miss. The future of food safety depends on mNGS since it offers a quicker and more thorough method of monitoring food safety, preventing outbreaks, lowering food recalls, and safeguarding public health. Because it can quickly identify the source of foodborne pathogens and help contain outbreaks before they worsen, mNGS is essential to food safety. Every minute matters in stopping the spread of foodborne illness once identified. By rapidly analysing contaminated food, production environments, and even infected patients, mNGS can pinpoint the precise source of contamination, in contrast to traditional methods that may take days or even weeks to isolate and identify the culprit. Public health officials can react quickly thanks to this speed and accuracy, implementing safety precautions, issuing targeted recalls, and halting the spread of dangerous bacteria like *Salmonella typhi, Escherichia. coli, and Listeria monocytogenes* [96]. mNGS helps protect the consumers and fortifies food safety laws by expediting outbreak investigations and response times, which lowers the possibility of widespread contamination and disease. mNGS offers a powerful and comprehensive approach to food safety by detecting multiple pathogens at once while also analysing the entire microbial ecosystem within food products. mNGS can simultaneously detect of dangerous bacteria, viruses, fungi, and parasites in food through a single test. By identifying spoilage organisms that may compromise food safety, this technology detects harmful microbes and aids in food quality monitoring. [97]. Scientists and food safety specialists can better control contamination, anticipate the emergence of new pathogens, and improve food production processes by using mNGS, which offers a deeper understanding of microbial communities. In addition to preventing foodborne illness, this keeps food safer and fresher for customers. Foodborne pathogens cause approximately 600 million illnesses and 420,000 fatalities globally each year, highlighting the significant risk that contaminated food poses to public health [98]. These conditions, which are more common in young children, the elderly, and people with compromised immune systems, can range from minor digestive problems to serious, life-threatening infections. Contaminated food and water can rapidly spread outbreaks of *Salmonella Typhimurium, Escherichia Coli, Listeria monocytogenes* and other dangerous microorganisms, resulting in hospital stays, long-term health issues, and even death. Modern tools like mNGS are essential for more rapid and precise pathogen identification, which lowers the burden of foodborne illnesses and potentially saves lives. In conclusion, mNGS has great potential to improve food safety by offering sensitive, thorough, and quick pathogen detection. Its use in food safety is anticipated to grow as costs come down and technology improves, providing better early detection and control of foodborne pathogens.

ii. Shelf-Life Prediction

With mNGS, the genetic composition of the microbial communities found in food products is analysed. This technology detects and measures the microorganisms that cause spoiling or deterioration in quality. By examining these microbial profiles, researchers may predict the change of microbial activity under various storage conditions, which directly impacts shelf life. This information is essential for improving shelf life, decreasing food waste, and ensuring the products are delivered to customers in the best possible condition. Food manufacturers can use this modern technology to improve preservation methods and quality control procedures, resulting in safer, fresher, and longer-lasting food products. A study on the application of NGS in food microbiota analysis showed how effective this technology is at demonstrating the complexity of microbial communities found in food products [99]. NGS can simultaneously analyse thousands to millions of genetic sequences, providing an in-depth understanding of the microbial communities in food, in contrast to traditional methods that can only identify a small number of microbes at a time. Thus, scientists can identify not only dangerous pathogens but also good bacteria, spoilage organisms, and new microorganisms that could affect the quality and safety of food. On top of that, there is also a study of microbial ecology in fermented foods that has been proved to utilise mNGS technology, which provides important new information on microbial interactions and fermentation processes. NGS offers a comprehensive inventory of microbial operons and genes by examining the entire genetic composition of microbial communities, which aids researchers in comprehending the various microbes' functional roles during fermentation [99], [100]. With this increased knowledge, researchers can pinpoint the main bacterial and fungal species contributing to flavour development, texture, and preservation. They can also improve fermentation conditions to produce higher-quality products. These studies also show how microbial populations change over time, which helps industries that make kimchi, cheese, yoghurt, and other fermented foods better control their fermentation processes. The integration of this method with other technologies can also be helpful in the food manufacturing industry, especially in predicting food spoilage. Food safety and quality monitoring can be enhanced by integrating sensors based on nanotechnology with mNGS. These cutting-edge sensors can identify dangerous pathogens, chemical contaminants, and environmental changes in food items, giving real-time information on contamination and freshness. Food manufacturers and supply chain managers can monitor food quality during storage and transit by connecting these sensors to RFID systems [101]. Better decisions about product handling and distribution are made possible by the frequent freshness reviews made possible by this smooth integration technology. Managers can lower the risk of foodborne illnesses by optimising logistics and inventory management with access to these real-time insights. This ensures that only the freshest and safest products reach consumers.

O. Biosurveillance and Biothreats Detection

By providing a highly sensitive and objective method of directly identifying pathogens from clinical and environmental samples, mNGS has revolutionised biosurveillance and biothreat detection. Researchers and public health officials can track the development of pathogens, track the spread of infectious diseases, and find genetic markers linked to drug resistance with the help of mNGS, which offers comprehensive genomic information. This capability is vital when dealing with quickly changing threats like pandemic viruses, bacteria that are resistant to antibiotics, and possible bioterrorism agents. mNGS is influencing the future of global health security by facilitating quicker reactions to biological threats and enhancing readiness for newly emerging infectious diseases through its real-time analysis of complex microbial communities.

i. Environmental Monitoring

By examining the microbial communities in soil, water, and air, mNGS plays a critical role in environmental monitoring by offering important insights into the stability and health of ecosystems. Scientists can find early indicators of environmental stressors like pollution, climate change, or habitat disruption by using mNGS to identify changes in microbial populations. For example, abrupt changes in soil microbiota may indicate nutrient imbalances or pesticide overuse. In contrast, abrupt changes in microbial diversity in water sources may indicate chemical contamination or toxic algal blooms. This technology aids in developing strategies to safeguard ecosystems and human health by providing a comprehensive and real-time view of microbial dynamics. Through microbial diversity analysis and detecting possible contaminants, mNGS is an effective method for water quality monitoring in aquatic environments. To determine pollution levels and guarantee the security of drinking water sources, it can detect bioindicators of sewage contamination. Studies employing Illumina MiSeq sequencing have demonstrated how the microbial communities in reservoirs vary with the seasons, offering valuable information on variations in water quality and assisting in the more efficient management of water supplies [102]. A deeper understanding of how microbes degrade pollutants and contribute to natural water purification can be acquired by mNGS's capability to identify genes associated with toxin production and biodegradation. By revealing these microbial processes, mNGS contributes significantly to preserving water supplies, enhancing wastewater treatment methods, and guaranteeing everyone access to safe and clean water. To sum up, mNGS provides a reliable method for analysing microbial communities in various settings, identifying variations suggestive of environmental stressors or contamination, and detecting pathogens and pollutants. It offers important insights into ecosystem dynamics and health and is used in everything from environmental monitoring to clinical diagnostics.

ii. Epidemiological Surveillance

mNGS is improving epidemiological surveillance by making it possible to quickly and thoroughly identify pathogens in environmental and clinical samples. Due to limited access to traditional diagnostic tools and specialised laboratories, this technology is beneficial in low- and middle-income countries (LMICs). Because mNGS can simultaneously detect known, emerging, and unexpected infectious agents in a single test, it is a valuable tool for outbreak detection and response, in contrast to conventional methods that frequently require a prior understanding of the targeted pathogen. In areas with a high disease burden and limited resources, mNGS offers a scalable and affordable method for identifying pathogens, tracking the spread of disease, and guiding public health initiatives. mNGS assists LMICs in strengthening their disease surveillance systems, enhancing healthcare readiness, and ultimately saving lives through earlier and more effective interventions by filling the

diagnostic gap. Because it can quickly identify the pathogens causing disease outbreaks, mNGS is essential for outbreak investigations and allows for quick public health responses. By identifying known and unknown infectious agents in a single sample, mNGS offers a comprehensive and unbiased method. During the 2013–2016 Ebola outbreak in West Africa, mNGS was utilised to map the virus's transmission patterns in real time and track its origin, providing a potent illustration of its impact [103]. By doing away with the need to send samples to faraway labs, this technology greatly shortened turnaround times and facilitated faster responses from health officials. mNGS aids in more efficient outbreak containment, halting further spread and ultimately saving lives by speeding up pathogen detection and genomic surveillance. Another example can be found in Uganda, indicating that mNGS was utilised to analyse cerebrospinal fluid (CSF) samples from 368 HIV-infected people. The results showed how well the technology detected a variety of pathogens. Infections that might have gone unnoticed with conventional diagnostic techniques, such as Nocardia brasiliensis and Wesselsbron virus, were among the 81 distinct infectious agents the researchers discovered [104]. The study also demonstrated the range of co-infections that mNGS can detect, including toxoplasmosis and tuberculosis, which are prevalent but challenging to diagnose in immunocompromised patients. In vulnerable patient populations, where prompt and precise detection is essential for successful treatment and better results, mNGS improves the diagnosis of complex infections by offering an in-depth overview of the microbial environments in one laboratory test.

To sum up, mNGS has the potential to enhance disease surveillance by eliminating the necessity of labour-intensive and time-consuming traditional culture-based methods. The ability of mNGS to quickly identify various pathogens from a single sample makes it an effective tool for early disease detection and outbreak response. To fully utilise this technology, several issues need to be resolved in LMICs. Processing vast amounts of sequencing data requires trained staff and easily accessible bioinformatics pipelines, while technical support, data exchange, and resource sharing require robust collaborative networks. mNGS can develop into a broadly available instrument for global health surveillance by investing in infrastructure, training, and international collaborations. This will enhance public health readiness globally and aid in the more effective detection of new threats.

V. RECENT ADVANCES AND TRENDS

mNGS has become a game-changer in detecting pathogens and predicting drug resistance. With faster processing, higher efficiency, and greater flexibility, it provides a more comprehensive and unbiased approach to identifying infectious agents, leading to quicker and more accurate diagnoses [105], [106], [107], [108], [109], [110].

P. Improved Throughput and Speed

Second-generation sequencing, also known as NGS or high-throughput sequencing, improved DNA sequencing by overcoming the low throughput of first-generation methods. Since the early 2000s, advances in massively parallel sequencing have made large-scale sequencing faster and more accessible. Innovations like pyrosequencing, reversible terminator chemistry, and oligonucleotide ligation have dramatically increased sequencing speed while significantly reducing costs over time [111]. In diagnostic microbiology labs, NGS is widely used for two main purposes. One is targeted sequencing, where specific genetic regions are enriched using amplification or probe hybridization. The other is mNGS, which provides a broader, more comprehensive approach to detecting pathogens. Both techniques help improve the accuracy and speed of microbial identification and disease diagnosis [112]. Advancements in high-throughput sequencing have significantly improved the accuracy of long-read sequencing by reducing errors and enhancing reliability [113], [114]. While short-read sequencing is highly effective for tasks like measuring gene expression, identifying genetic variants, and analysing the abundance of specific sequences, it has limitations in capturing the full genome complexity. Long-read sequencing, on the other hand, provides a more complete and detailed view, allowing researchers to identify intricate structural variations such as large insertions, deletions, inversions, and duplications. This capability is especially valuable for studying complex genetic disorders and uncovering hidden variations that might be missed with shorter reads [113], [114], [115]. mNGS is significantly more effective than traditional diagnostic methods, especially when detecting tuberculosis (TB) and rare infections. For example, in one study, mNGS identified 67.23% of TB cases within just three days, whereas nearly half of the cases detected using conventional methods took over 90 days to confirm [116]. This dramatic difference in turnaround time can be lifesaving, as faster diagnosis means earlier treatment and a lower risk of disease progression or transmission. Beyond TB, some infections also have extremely low culture-positive rates or are so uncommon that there are few clinical precedents to guide diagnosis. Traditional lab tests might miss these infections or take weeks to return results, delaying crucial treatment. However, mNGS can rapidly and accurately identify these elusive pathogens, giving clinicians the information they need to make timely, targeted treatment decisions. By offering both speed and accuracy, mNGS is revolutionizing infectious disease diagnostics, making it especially valuable in complex, undiagnosed, or high-risk cases where traditional methods often fall short [110].

Q. Unbiased Sampling

mNGS is a breakthrough in pathogen detection, allowing researchers and clinicians to identify known and novel infectious agents without needing prior assumptions about the culprit. This unbiased approach provides a more complete picture of microbial communities, making it especially valuable in clinical diagnostics. Using high-throughput sequencing, mNGS can analyse genetic material directly from patient samples, detect expected pathogens, uncover unexpected ones, and even discover entirely new organisms. This makes it far more powerful than traditional culture-based methods, offering a deeper and more accurate understanding of infections [117]. mNGS utilizes shotgun sequencing to analyse all genetic material present in a sample. This approach allows researchers to examine total nucleic acids for DNA and RNA without prior knowledge of a specific pathogen. As a result, mNGS can simultaneously detect a wide range of infectious agents, including bacteria, viruses, fungi, and parasites, all from a single test. This comprehensive method is particularly valuable in clinical diagnostics, where traditional tests might miss co-infections or rare pathogens. By providing a more complete picture of microbial communities, mNGS enhances our ability to diagnose complex infections, track disease outbreaks, and guide more effective treatment strategies [117]. As global demographics shift and climate change continues to influence disease

patterns, the need for comprehensive and scalable tools to monitor and identify emerging pathogens has become more urgent. A robust public health response relies on rapidly detecting and tracking infectious agents, particularly those responsible for new and re-emerging diseases. mNGS offers a powerful, unbiased approach to identifying the causative agents of infections from a wide range of clinical samples. This technology has the potential to revolutionize infectious disease surveillance by enabling early detection, improving outbreak response, and even aiding in the prediction and prevention of future epidemics [118].

R. Surveillance and Prediction Drug-Resistance

mNGS can potentially transform respiratory infection diagnostics by overcoming the limitations of traditional culture-based methods. Unlike conventional approaches, mNGS can identify pathogens directly from patient samples without the need for cultivation, while also providing insights into the host's immune response to infection [119]. In theory, mNGS could also help predict antimicrobial resistance (AMR) by detecting bacterial resistance genes. While whole-genome sequencing of cultured bacterial isolates has been well studied [120], much less is known about how well mNGS can directly detect AMR genes in respiratory samples [121], [122], [123], [124], [125]. One of the main challenges in using mNGS for AMR prediction is that resistance genes are often present in very low amounts in respiratory and other clinical fluids, making them difficult to detect with standard sequencing methods [121]. A promising solution to this problem is CRISPR/Cas9-based targeted enrichment, specifically the FLASH (Finding Low Abundance Sequences by Hybridization) method, which enhances the detection of low-abundance AMR genes in clinical samples. However, further validation in real-world clinical settings has been needed to confirm its effectiveness [126].

This study aims to bridge these gaps by analysing a cohort of critically ill patients. We evaluate the potential of both DNA and RNA-based mNGS for predicting AMR in bacterial pathogens causing lower respiratory tract infections (LRTIs). Additionally, we explore how mNGS can support AMR surveillance and enable the rapid detection of clinically significant resistance genes by integrating CRISPR/Cas9 enrichment with real-time nanopore sequencing [126]. Next, by analysing wastewater, livestock, and environmental samples, mNGS also enables the early detection of emerging drug-resistant pathogens, helping monitor how resistance spreads within communities before it becomes a significant clinical problem. This approach allows scientists to track outbreaks, detect new variants of resistant bacteria and viruses, and assess the effectiveness of infection control measures [127]. Wastewater monitoring, in particular, serves as a valuable checkpoint for identifying resistance hotspots and evaluating how well treatment plants prevent antibiotic-resistant bacteria from entering the environment. By providing a comprehensive view of microbial resistance patterns, mNGS helps guide antibiotic stewardship programs, infection prevention strategies, and public health policies. Its ability to stay ahead of drug-resistant threats makes it an essential tool in the fight against antimicrobial resistance, ensuring a more proactive approach to protecting public health [127].

S. Enhanced Sensitivity and Versatility

mNGS is transforming infectious disease diagnostics with its high sensitivity and unparalleled versatility. Its high sensitivity allows for detecting low-abundance or unculturable microbes, making it especially valuable in immunocompromised patients, sepsis cases, and encephalitis of unknown origin. With its versatility, mNGS can analyse diverse sample types such as blood, CSF, BALF, tissue, and even provide insights into AMR and microbiome composition, paving the way for precision infectious disease management. mNGS is highly accurate in detecting gut infections, with a sensitivity between 89.2% and 100%, meaning it catches nearly all cases of pathogens such as bacteria, viruses, fungi, and parasites. This makes it more reliable than traditional primer-based PCR and culture methods, giving clinicians a better chance of identifying infections quickly and accurately [128]. One of the most significant advantages of mNGS is its ability to work with a wide range of sample types, including blood, stool, cerebrospinal fluid CSF, and more. This versatility makes it incredibly useful in different clinical situations, from detecting bloodstream infections and gastrointestinal diseases to diagnosing meningitis and encephalitis. Since mNGS does not rely on traditional culture methods, it can be especially valuable when dealing with hard-to-diagnose infections, immunocompromised patients, or cases where standard tests come back inconclusive. By providing a comprehensive view of the microbial landscape, mNGS helps clinicians make faster, more accurate diagnoses across multiple fields of medicine [129].

Identifying microbes through mNGS is not just about sequencing, it also depends on the bioinformatics tools and databases used to analyse the data. These tools work by classifying sequencing reads into different microbial taxonomies, but they vary in their algorithms and the reference databases they rely on. As a result, the choice of bioinformatics pipeline can greatly impact sensitivity and accuracy, potentially leading to variations in pathogen detection. Studies comparing different mNGS classification methods have shown inconsistencies in results, particularly in how pathogens are identified and how their relative abundance is measured [130], [131]. This variability poses a challenge for clinical use, where accurate and reproducible data is essential for making informed treatment decisions. To fully harness the power of mNGS in infectious disease diagnostics, there is a growing need to standardize bioinformatics workflows. By improving consistency in pathogen classification and ensuring more reliable microbial profiling, these advancements will help make mNGS a more trusted and widely adopted tool in clinical settings [131]. Achieving full genome coverage can be particularly difficult for low-biomass samples, where genetic material is limited. This poses a challenge in microbial identification, as incomplete sequencing data may lead to missed or misclassified pathogens. Additionally, the choice of database used for taxonomic classification plays a crucial role, as different reference databases can yield varying levels of accuracy in identifying and categorizing microbial species. Inconsistent or incomplete classification can result in misinterpretation of results, affecting clinical decision-making. To enhance analytical sensitivity and improve detection in these challenging cases, using high-output sequencing platforms like Illumina HiSeq, NovaSeq, or Oxford Nanopore PromethION can be beneficial. These platforms generate substantially larger sequencing datasets, increasing the chances of detecting low-abundance pathogens and providing more comprehensive genome coverage. This, in turn, improves the accuracy of taxonomic classification and enhances the reliability of results. By combining high-yield sequencing technologies with well-curated databases, researchers and clinicians can significantly improve pathogen detection and ensure more precise and actionable diagnoses in cases where traditional methods may fall short [132].

VI. FUTURE DIRECTIONS

The future directions of mNGS in pathogen identification focus on overcoming existing limitations, standardizing workflows, and enhancing phenotypic prediction. As mNGS transitions from research to clinical applications, it presents unique advantages and challenges that must be addressed for broader implementation.

T. Overcoming Limitations

mNGS is becoming increasingly widespread due to rapid technological advancements and improved accessibility. Its ability to provide unbiased, comprehensive pathogen detection has proven invaluable in preventing, diagnosing, treating, and tracking infectious diseases. Numerous studies and real-world cases have demonstrated its success in identifying infections that traditional methods might miss. However, despite its promise, challenges remain before mNGS can be fully optimized for routine use. One major hurdle is data interpretation [133]. Unlike conventional diagnostic tests targeting specific pathogens, mNGS detects all microbial DNA in a sample, often revealing hundreds or thousands of different microorganisms. Among these, pinpointing the causative agent responsible for a patient's illness is not always straightforward. Factors such as background contamination, microbial colonization, and the presence of non-pathogenic microbes can complicate interpretation [133]. This challenge highlights the need for more refined analytical approaches to enhance mNGS results' specificity. The accuracy of pathogen detection in sequencing depends heavily on the technology and databases used [134]. Several factors can impact the results, including incomplete or outdated reference databases, sequencing errors, misclassified organisms, and contamination within the database. If a database contains incorrectly labeled sequences or biases in its reference data, it can lead to misidentification of pathogens or missing key infections altogether [134], [135]. Ensuring high-quality, well-curated reference databases is crucial for improving the reliability and accuracy of sequencing-based diagnostics.

As sequencing technology advances and becomes more cost-effective, there is a growing demand for affordable, widely accessible sequencing assays to improve infectious disease diagnosis, patient care, and public health efforts. While mNGS provides a broad, unbiased approach to pathogen detection, it also comes with high costs, data complexity, and difficulty distinguishing key pathogens from background microbial sequences. To overcome these limitations, targeted next-generation sequencing (tNGS) is gaining traction as a more practical and efficient alternative. Unlike mNGS, tNGS focuses specifically on enriching target pathogens in patient samples using multiplex PCR amplification or probe capture techniques, leading to enhanced detection sensitivity [136]. This targeted approach improves pathogen identification accuracy while reducing sequencing costs and data complexity. Clinical studies have demonstrated that tNGS achieves a high positivity rate in respiratory samples and maintains strong sensitivity (70.8%–95.0%), even in low-pathogen-load samples such as blood and cerebrospinal fluid. Beyond detection, tNGS is proving to be a powerful tool in antimicrobial resistance surveillance, particularly in identifying drug-resistant strains of MTB. It enables the detection of resistance-associated genes, providing clinicians with critical insights for guiding treatment decisions with precision that is difficult to achieve with mNGS alone. As sequencing technologies continue to evolve, tNGS is emerging as a valuable tool in clinical microbiology, offering a cost-efficient, high-sensitivity approach to infectious disease diagnostics and antimicrobial resistance monitoring [136]. By integrating both mNGS and tNGS in diagnostic workflows, the future of precision medicine in infectious disease management is becoming increasingly promising. Despite its advanced sequencing capabilities, mNGS still has data interpretation, cost, and sensitivity limitations that require further refinement.

U. Workflow Standardisation

As personalized medicine advances, laboratory-developed tests (LDTs) play a crucial role in enhancing clinical diagnostics and treatment strategies. These customized tests enable laboratories to integrate cutting-edge technologies to meet specific patient needs, leading to more precise and individualized medical care. Among these emerging technologies, mNGS is a powerful tool for infectious disease diagnostics. However, its complex workflow, high technical requirements, and the need for precise interpretation pose significant challenges. Its application within LDTs must be carefully regulated and standardized to ensure accuracy, reliability, and patient safety. Due to the high technical threshold of mNGS platforms and the intricate processes involved in analysing sequencing data, experts from various fields including infectious diseases, clinical diagnostics, critical care, and in vitro diagnostics have collaborated to develop a standardized framework for integrating mNGS into LDTs [137].

Despite these efforts, no universally accepted standard for analysing and interpreting mNGS results remains. Variability in sequencing platforms, bioinformatics pipelines, and reference databases can lead to inconsistent conclusions, even when examining the same sample. This lack of standardization remains a significant hurdle in clinical adoption and regulatory approval. To address these challenges, researchers are working to enhance bioinformatics tools, refine reference databases, and establish clear interpretation guidelines. These improvements are essential to ensure that mNGS not only detects pathogens but also identifies the right one, making it a more reliable and actionable tool for guiding patient care [133]. The newly developed consensus guidelines aim to tackle these challenges by establishing key quality standards for mNGS-based LDTs. These guidelines include workflow standardization, performance validation, quality control measures, and clinical report interpretation. By implementing these recommendations, the goal is to optimize the use of mNGS, improve diagnostic accuracy, and enhance patient outcomes, all while maintaining high medical safety standards [137]. With continued technological advancements and improved regulatory frameworks, mNGS is expected to become integral to modern infectious disease diagnostics, bridging the gap between cutting-edge sequencing technology and real-world clinical application to improve public health and patient care.

V. Enhancing Phenotypic Prediction

The advancement and growing use of mNGS have significantly improved how infectious diseases are diagnosed. This cutting-edge technology allows for detecting nearly all potential pathogens in a clinical sample, making it especially valuable in cases where traditional diagnostic methods fall short. Beyond simply identifying pathogens, mNGS can also uncover crucial genomic information, such as the presence of ARGs, which helps predict how a pathogen might respond to different treatments. However, despite these advancements, there remains a significant gap in accurately predicting antibiotic resistance and susceptibility directly from mNGS data. Current methods often struggle with low sensitivity, incomplete reference databases, and difficulty distinguishing between genetic

resistance markers and actual phenotypic resistance. This limitation poses a challenge for clinicians who need reliable, real-time data to guide effective antibiotic treatment decisions. Researchers are developing more advanced computational tools and predictive models to fully harness the potential of mNGS in AMR surveillance and precision medicine. These improvements aim to enhance the accuracy and reliability of resistance predictions, ensuring that mNGS can not only detect infections quickly but also help guide appropriate and targeted antibiotic therapies, ultimately improving patient outcomes and combating the global threat of antibiotic resistance [138].

i. Machine Learning Models

Machine learning (ML) has the potential to outperform current tools in predicting how genes translate into traits, thanks to its ability to recognize patterns and relationships in complex data independently [139]. The reliability of machine learning in detecting antibiotic resistance heavily depends on the quality and diversity of the data it is trained on. The predictions may be less accurate or misleading if the dataset lacks enough genetic variation or does not accurately represent real-world infections. Ensuring that machine learning models are built on comprehensive, diverse datasets is crucial for improving their ability to identify resistance patterns and guide effective treatments accurately [140]. ML has become a major focus in computer science, especially with the rise of deep learning and neural networks. Supervised ML, particularly classification, remains a key area of research, and interest in deep learning has grown significantly over the past two years, which is a trend that is likely to continue [141]. These technologies have greatly improved our ability to detect pathogenic microorganisms, aiding in drug discovery, deepening our understanding of infections, and speeding up diagnostic development. For example, AI-driven machine learning is crucial in this progress, particularly in image analysis, genome sequencing, and natural language processing for pathogen identification. By enhancing diagnostic accuracy and efficiency, AI supports early detection, personalized treatment, and overall public health safety [142].

ii. Enrichment Methods

One strategy for improving pathogen detection in mNGS is host depletion, which helps enrich microbial sequences by reducing the overwhelming presence of human genetic material. Several approaches have been developed, each targeting different biological components. For instance, differential lysis techniques selectively break down human cells while preserving those of bacteria and fungi, increasing their relative abundance in the sample [143]. Another widely used method involves removing excessive human ribosomal and mitochondrial RNA using antibody hybridization or depleting abundant sequences through hybridization techniques such as depletion of abundant sequences by hybridization (DASH) [144]. Additionally, nuclease treatment before DNA/RNA extraction selectively degrades human nucleic acids while leaving encapsulated viral genomes intact, making it particularly useful for enriching viral sequences [145], [146]. While these approaches enhance microbial detection, they can also introduce biases in sequencing results. For example, differential lysis favours detecting bacteria and fungi with robust cell walls [143]. At the same time, nuclease treatment before extraction primarily enriches encapsulated viruses, potentially overlooking pathogens that do not fit these profiles [145], [146]. Host depletion can also be performed at the RNA level using non-human primers, which selectively reduce the presence of human ribosomal or mitochondrial RNA, further refining microbial signal detection [147], [148]. These techniques have been successfully applied to sample types with high human RNA content, such as cellular lysates and throat swabs, where removing excess host genetic material significantly improves microbial detection. However, their effectiveness in cell-free fluids such as plasma, CSF, and serum remains uncertain, as these samples naturally contain lower amounts of human ribosomal RNA. Additionally, the impact of using non-human primers on the detection sensitivity for non-viral pathogens or on the proportion of contaminant sequences remains unclear, highlighting the need for further research to optimize host depletion strategies across different sample types [149].

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