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REVIEW

# The application status of sequencing technology in global respiratory infectious disease diagnosis

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## Abstract

Next-generation sequencing (NGS) has revolutionized clinical microbiology, particularly in diagnosing respiratory infectious diseases and conducting epidemiological investigations. This narrative review summarizes conventional methods for routine respiratory infection diagnosis, including culture, smear microscopy, immunological assays, image techniques as well as polymerase chain reaction(PCR). In contrast to conventional methods, there is a new detection technology, sequencing technology, and here we mainly focus on the next-generation sequencing NGS, especially metagenomic NGS(mNGS). NGS offers significant advantages over traditional methods. Firstly, mNGS eliminates assumptions about pathogens, leading to faster and more accurate results, thus reducing diagnostic time. Secondly, it allows unbiased identification of known and novel pathogens, offering broad-spectrum coverage. Thirdly, mNGS not only identifies pathogens but also characterizes microbiomes, analyzes human host responses, and detects resistance genes and virulence factors. It can complement targeted sequencing for bacterial and fungal classification. Unlike traditional methods affected by antibiotics, mNGS is less influenced due to the extended survival of pathogen DNA in plasma, broadening its applicability. However, barriers to full integration into clinical practice persist, primarily due to cost constraints and limitations in sensitivity and turnaround time. Despite these challenges, ongoing advancements aim to improve cost-effectiveness and efficiency, making NGS a cornerstone technology for global respiratory infection diagnosis.

**Keywords** Next-generation sequencing (NGS) · Respiratory infectious diseases · Metagenomic next-generation sequencing (mNGS) · Sequencing technology

## Introduction

Next-generation sequencing (NGS) enables massively parallel processing of thousands to billions of DNA fragments, thus it is a huge leap forward compared to conventional diagnostic methods for respiratory infectious diseases. A key application of NGS in clinical microbiology is metagenomic NGS (mNGS), allowing unbiased detection of pathogens directly from patient specimens. This narrative review focuses on the utilization of sequencing technology in diagnosing global respiratory infectious diseases [1–10]. In contrast to targeted approaches employing polymerase chain reaction (PCR), primer extension or probe enrichment which restrict detection to specific organisms in clinical respiratory specimens, untargeted mNGS employs shotgun sequencing to randomly survey analyte DNA/RNA masse. While NGS has diverse applications in sequencing respiratory tract samples for typing, epidemiology, predicting antimicrobial susceptibility and characterizing virulence factors

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[11, 12], as well as evolutionary tracing [13], resistance testing [14–16] and microbiome studies [17], this narrative review examines the latest advances in clinical mNGS for infectious diseases. Although existing reviews have described NGS technologies [18, 19] and applications in infectious diseases [13, 16, 20], rapid progress in this fledgling field warrants updated discussion.

## Conventional diagnosis methods of respiratory infectious disease

The traditional diagnostic methods for respiratory infectious diseases include culture, smear microscopy, immunological assays, imaging techniques, and nuclear acid detection methods. These conventional techniques are still vital for identifying respiratory infections and underscore the importance of incorporating advanced technology. The following is a brief overview of some of the traditional testing methods.

### Culture and smear microscopy

#### Culture

Bacterial culture, the gold standard for detecting respiratory pathogens, is labor-intensive, requires expertise, and has a long turnaround time. Traditionally, direct virus detection has been accomplished through cell culture method [21], which also serve as the only reference laboratory method capable of proving viral infectivity. Cell culture, used for virus detection, takes 3–5 days for diagnosis and require robust sample storage [21], affecting sensitivity [22, 23] and increasing costs [24]. Bacterial culture isolates can also be utilized as samples for subsequent sequencing-based diagnostics.

#### Smear Microscopy

In bacterial detection, microscopic examination can assess sample quality, detect contamination, provide early infection indicators and guidance for treatment as well [25–27]. It is crucial for virus detection and identifying new strains causing outbreaks [25, 28–30]. However, it still has limitations such as high labor intensity, massive time consumption, high skill requirements for technical personnel, high requirements for experimental conditions and virus particle concentration, and long turnover time (3–16 h) [30–32].

### Immunological assays

#### Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) detects antibodies or antigens through specific enzyme markers. It is rapid, accurate, reliable, and affordable, widely used in respiratory virus detection. Variants include direct, indirect, sandwich, competitive, and competitive inhibition ELISA.

#### Immunofluorescence (IF)

Direct Immunofluorescent Assay (DFA) detects respiratory viruses like influenza from nasopharyngeal samples but cannot identify subtypes and varies in sensitivity [33]. Fluorescent Antibody Assay (IFA) is widely used in the detection of influenza viruses, parainfluenza viruses, respiratory syncytial viruses and other viruses, and IFA is more specific and sensitive [34]. Fluorescent Immunochromatographic Assay (FICA) is highly sensitive, specific, and suitable for rapid and high-throughput detection.

#### Chemiluminescence Immunoassay (CLIA)

Chemiluminescence Immunoassay (CLIA) is highly sensitive and fast, used for respiratory disease monitoring. The CLIA method has a high degree of specificity, but it requires special equipment, high operational skills, and high detection costs, which require further improvement and development [35].

#### Lateral Flow Immunoassay (LFIA)

Lateral Flow Immunoassay (LFIA) is a mature, rapid detection method used in various on-site tests or point-of-care (POC) tests [36]. The most commonly used tracer in the LFIA method is colloidal gold, namely the Colloidal Gold Lateral Flow Immunochromatography Assay (GICA). GICA is sensitive, specific, simple, fast, and ideal for large-scale respiratory virus detection.

### Image techniques

#### Chest X-Ray (CXR)

When laboratory testing resources are scarce, chest x-ray (CXR) and computed tomography (CT) have an important complementary effect on laboratory testing of the respiratory virus, and some abnormalities could be found in imaging tests before early symptoms appear.

#### Computed tomography (CT)

Computed tomography (CT) can detect fine structures and small lesions of the lungs, which is of great significance in respiratory viral infectious diseases. It is highly sensitive but has high radiation doses, costly, and requires skilled operators, which has a partial impact on the popularization of CT in respiratory virus diagnosis [37].

#### Ultrasound (US)

Ultrasound (US) is non-invasive, suitable for dynamic monitoring, especially in special groups like pregnant women. Point-of-care ultrasound (POCUS) and conventional lung ultrasound (LUS) are widely used in respiratory viral infectious diseases but less accurate than CXR and CT.

### Methods based on nucleic acid detection

#### Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is sensitive, simple, and rapid [38]. It is considered as the gold standard for the detection of SARS-CoV-2 viruses due to its ability to amplify small amounts of viral genetic material from the sample. Additionally, multiplex real-time PCR platforms are increasingly prevalent in laboratories, primarily due to enhanced diagnostic sensitivity and reduced turnaround time [39]. PCR has high diagnostic value but also limitations in excluding infection and risk of false negatives. Thus it should not be used as the sole basis for patient diagnosis.

#### Isothermal amplification (IA)

Loop-mediated Isothermal Amplification (LAMP) is fast, easy, and suitable for unpurified samples. In addition, LAMP requires a lower temperature than PCR, which is easier to combine with RT reactions (i.e., RT-LAMP), allowing direct detection of target RNA without a separate RT step, greatly reducing the total reaction time. Therefore, LAMP is a powerful tool for point-of-care diagnosis with a wide range of applications [40].

Transcription-mediated amplification (TMA) is a patented single-tube isothermal amplification technique that uses retroviral replication as a model to amplify specific regions of RNA or DNA more efficiently than RT-PCR [41]. Clinical studies at the Medical Center of Göttingen University confirm that Hologic Panther TMA platform is suitable for fully automated, high-throughput SARS-CoV-2 screening [42].

Recombinase polymerase amplification (RPA) is able to search for homologous sequences in double-stranded DNA by using recombinase to form protein-DNA complexes with oligonucleotide primers. Once the homologous sequence is localized, a strand displacement reaction occurs to form and initiate DNA synthesis, exponentially amplifying the target on the template. Single-stranded DNA-binding (SSB) proteins bind to displaced DNA strands and form stable D-loops, preventing further substitution. The entire amplification response is rapid, starting with a few target DNA bp and reaching detectable levels within a few minutes [43].

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a family of nucleic acid sequences found in prokaryotes such as bacteria. Certain enzymes in the Cas12 and Cas13 families can be programmed to target and cleave viral RNA sequences [44]. In recent years, CRISPR/Cas12a, CRISPR/Cas13a, and CRISPR/Cas13b systems have been used to detect human pathogens (bacteria and viruses), they are a series of rapid and sensitive tests [45]. Compared to RT-qPCR, CRISPR offers significant advantages over RT-qPCR because it does not require thermal cycling and isothermal signal amplification, such as fast turnaround time, good target specificity of single nucleotides, and rapid diagnosis of samples from respiratory swabs within 40 min, replacing complex laboratory systems.

#### Nucleic acid hybridization based on microarray

Microarray detection can be performed for the detection of virus-specific nucleic acids [46]. In addition, microarray testing can also be used to identify SARS-associated mutations and has been used to detect up to 24 single nucleotide polymorphisms (SNPs) associated with SARS spike(S) gene mutations with 100% accuracy [47].

## The application status of sequencing technologies

### Introduction of sequencing technologies

#### Evolution of DNA sequencing technologies

The advent of Sanger sequencing in 1977 enabled the first generation of automated DNA sequencing [48], setting the standard for sequencing single genes and small genomes for over thirty years. However, its limited throughput and high cost made it impractical for large-scale sequencing applications. The introduction of next-generation sequencing (NGS) in the mid-2000s dramatically improved sequencing capabilities [49]. Platforms such as 454 pyrosequencing and Solexa sequencing performed massively parallel sequencing using a sequencing-by-synthesis approach [44]. Billions of sequencing reactions could be performed simultaneously,

overcoming the limitations of Sanger sequencing. The first NGS instruments increased throughput over Sanger sequencing by orders of magnitude, generating over 1 billion bases of sequence data in a run costing 1/10 as much [50]. Since then, successive advances have yielded continued improvements in output and cost efficiency. Key innovations include emulsion PCR, reversible dye terminators, zero mode waveguides, and semiconductor sequencing [51]. Modern high-throughput platforms like the Illumina NovaSeq can generate up to 6 Tb of data in < 2 days at just $500–600 per genome [52]. Long read technologies like PacBio and Oxford Nanopore provide reads over 10 kb to resolve complex regions [44]. These technological developments have enabled the widespread adoption of NGS across research and clinical settings.

The Illumina platforms (iSeq, MiSeq, MiniSeq, NextSeq, HiSeq, NovaSeq) are widely used for sequencing. They employ bridge amplification where single DNA molecules are attached and clonally amplified on a flow cell, analogous to microbial colonies on agar [53]. Sequencing is performed by sequential addition of fluorescently labeled nucleotides, followed by optical detection. A key limitation is barcode index switching between multiplexed libraries [54], which can generate false positive pathogen detections in mNGS. This is exacerbated on high-throughput platforms using patterned flow cells with exclusion amplification chemistry. The Ion Torrent system (Thermo Fisher) clones single DNA molecules onto beads in an emulsion [55]. Sequencing relies on pH changes induced by nucleotide incorporation for detection. The BGISEQ (BGI) platform utilizes DNA nanoballs and sequenced by synthesis with fluorescent detection, similar to Illumina [56]. Though utilized for clinical mNGS [57], it is not yet commercially available in the United States. Although widely used, the shorter read lengths of Illumina sequencing platform limit their applicability in clinical settings. However, Nanopore sequencing technology (NST) with its long read sequencing advantages has shown potential in overcoming this drawback. Oxford Nanopore produces portable sequencers using protein nanopores that sequence single-stranded DNA via electrical current changes [9]. Compared to sequence-by-synthesis approaches, nanopore sequencing is faster but has higher error rates and costs. It also does not require PCR amplification, though this is often still performed to achieve sufficient input levels (> 500 ng). The differences have implications on utility for clinical mNGS applications, particularly for diagnosing respiratory infection.

### Workflow of NGS

The NGS workflow typically consists of two components (Fig. 1). Firstly, there is the experimental work in the laboratory (wet lab), which includes sample preparation, DNA/RNA extraction, library preparation, and sequencing stages. The second process is the bioinformatics analysis (dry lab). There are two different NGS detection methods for sequencing pathogens' genome, namely targeted and untargeted sequencing, the purpose and method of these two sequencing are different.

The workflow of targeted NGS (tNGS) enriches specific genetic targets for sequencing and involves the following steps: (a) Target selection: Choose specific genes or genomic regions of interest for study, here referring to respiratory infectious disease pathogens, as targets. (b) Library preparation: Amplify the target regions in the sample using PCR to prepare the library. (c) Sequencing: Sequence the library using high-throughput sequencing platforms such as Illumina. (d) Library evaluation: Assess the concentration and quality of the library to ensure suitability for subsequent sequencing. (e) Data analysis: Analyze sequencing data to identify variations, mutations, or genetic markers in the targets. Targeted sequencing (i.e., amplicon NGS or tNGS). Amplicon NGS is commonly employed in clinical laboratories, allowing for high-throughput parallel sequencing of individual viral agent, which makes it suitable for outbreak investigations and public health surveillance scenarios [24, 58, 59].Besides, prior to targeted sequencing, targeted PCR amplification can enable sequencing at lower depths and facilitate extensive sample multiplexing, thereby reducing sequencing costs [60].

Metagenomic Next-Generation Sequencing (mNGS) sequences as much DNA and/or RNA from the sample as possible, involving the following steps: (a) Nucleic acid extraction: Extract nucleic acids (DNA/RNA) from the collected sample. (b) Construct sequencing libraries directly from the extracted nucleic acids without prior amplification of specific target regions. (c) Sequencing: Sequence the prepared libraries using high-throughput sequencing platforms such as Illumina. (d) Data analysis: Conduct bioinformatics analysis to classify the microbial composition present in the sample.

Bioinformatics pipelines for analyzing metagenomic NGS data have distinct requirements compared to pipelines detecting human mutations. A range of open-source and commercial software packages exist, including SURPI, Kraken and Taxonomer. Key analysis steps include: (a) Preprocessing raw reads - Removing sequencing adapters, low quality/complexity regions. (b) Host subtraction - Optionally aligning to human genome to filter out human reads. (c) Microbial classification - Aligning non-human reads to curated pathogen databases for taxonomic assignment. (d) Statistical analysis - Organizing and analyzing microbial assignments, with optional data visualization.

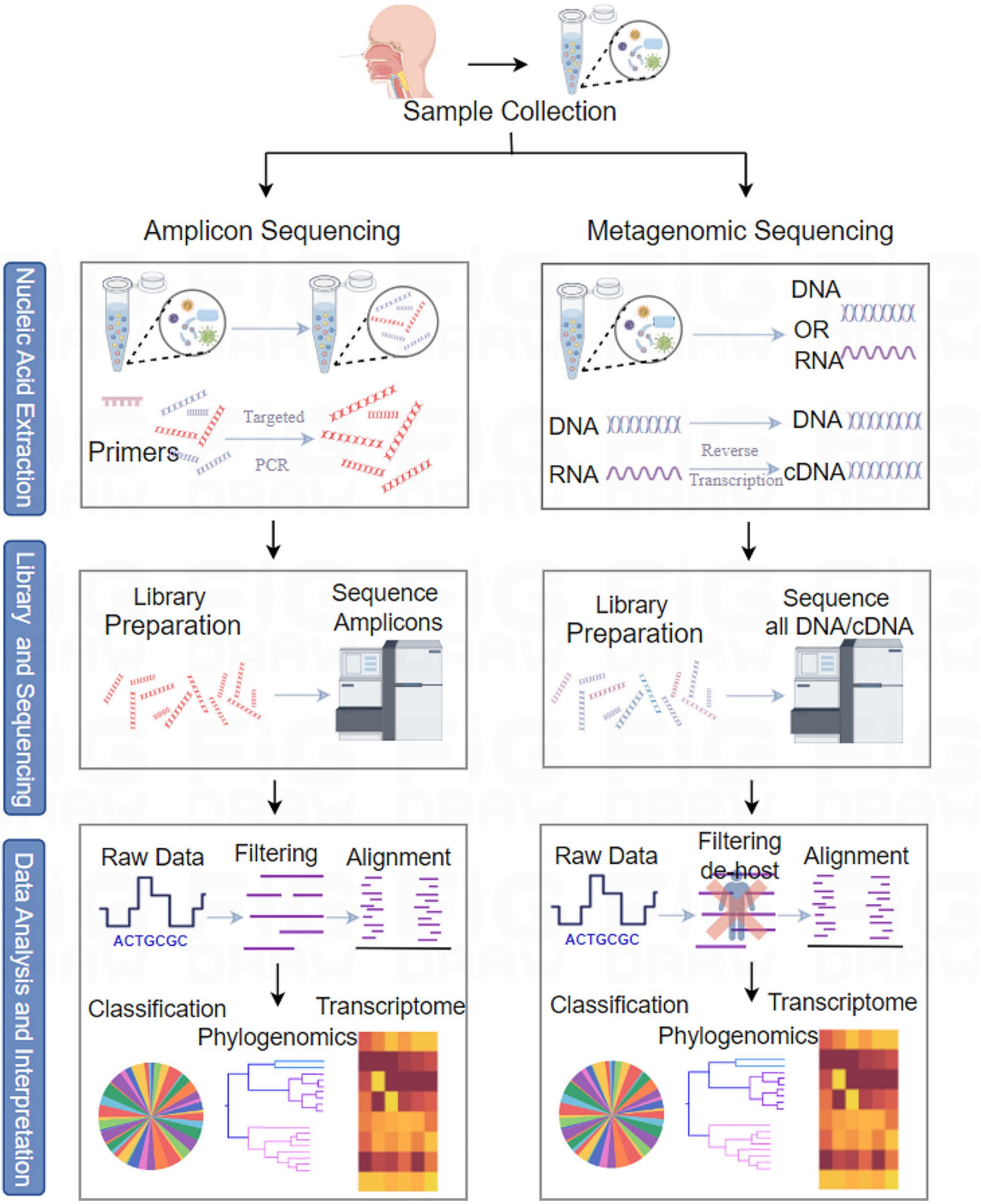


**Fig. 1** Workflow diagram of Amplicon and Metagenomic Next-generation Sequencing and Analysis. (By Figdraw)

Construction of comprehensive pathogen reference databases remains a challenge. A top-down approach starts from large databases like GenBank then excludes human and low-complexity sequences. Bottom-up approaches aggregate individually curated pathogen genomes. Databases can also be tailored to genomic regions specific for taxonomic classification. De novo assembly enables recovery of novel genomes not in reference databases. Standardized interpretation is difficult due to factors like incomplete/biased databases, genetic similarities between pathogens, and contamination risks. Robust bioinformatics workflows are needed, incorporating quality metrics, pathogen abundance estimates, expert reviews of unusual findings, and orthogonal confirmation. Continued improvements in metagenomic NGS bioinformatics will enhance test performance.

## Application status of NGS

Initial studies highlighted the potential of NGS for direct pathogen identification from clinical samples. In 2008, unbiased high-throughput sequencing characterized viruses causing hemorrhagic fever with renal syndrome [61]. A 2010 study used RNA sequencing to characterize the transcriptome of the 2009 H1N1 influenza pandemic strain in a patient respiratory sample, detecting viral sequences even at low viral loads amidst human background [62]. Single molecule sequencing identified mixed influenza variants in nasal swabs missed by RT-PCR [63]. These pioneering studies demonstrated the sensitivity of NGS for known and novel pathogens.

By 2011, NGS began to be applied for infectious disease diagnosis. A landmark study diagnosed neuroleptospirosis by sequencing cerebrospinal fluid, representing the first use of NGS for clinical diagnosis [3]. Subsequent studies further validated NGS for diverse pathogens including viruses, bacteria, fungi and parasites, with greater sensitivity than PCR or culture [64–66]. Targeted 16 S rRNA sequencing also effectively profiled bacteria in polymicrobial infections [67]. Oxford Nanopore sequencing rapidly characterized Ebola virus mutations during the 2014 outbreak [68]. These groundbreaking applications established NGS as a disruptive technology poised to transform clinical microbiology.

Since its debut in 2011, NGS has seen rapidly increasing usage for pathogen detection across diverse clinical settings as sequencing workflows and bioinformatics tools have improved [66, 69]. Key diagnostic applications include: (a) Identifying causative pathogens to guide treatment for infectious diseases, with utility shown for diagnosing encephalitis, meningitis, pneumonia and other syndromes, particularly where conventional testing is unrevealing [3, 20, 65]. (b) Outbreak investigation by rapidly sequencing pathogens to elucidate transmission dynamics and evolutionary trajectories, as demonstrated during recent Ebola, Zika and COVID-19 epidemics [68, 70, 71]. (c) Detecting antimicrobial resistance genes directly from specimens to guide effective antibiotic therapy [72, 73]. (d) Strain-level tracking of nosocomial infections to elucidate transmission patterns and inform infection control efforts [74]. (e) Cancer diagnostics by surveying tumor samples for oncogenic viruses [75]. NGS has been broadly applied on respiratory, blood, urine, cerebrospinal fluid, and tissue specimens [66, 69]. Plummeting costs have enabled adoption in clinical microbiology laboratories [76]. Here we mainly elaborate on the application of NGS in the detection related to respiratory infectious disease pathogens, including targeted and untargeted sequencing.

### Application of targeted sequencing (amplicon NGS)

While NGS methods have been widely applied in laboratory tests, many of its applications still remain confined to targeted sequencing only [24]. RNA sequencing has been effectively utilized for phylogenetic clustering of Zika virus infections. Analysis of the whole sequenced genome revealed a close relationship between the Zika virus circulating in Brazil and the sequence from French Polynesia [77], but limitations persist due to insufficient geographical coverage. Quick and his colleagues have successfully developed a real-time amplicon sequencing scheme that expands the geographic scope of detection by utilizing portable MinION sequencing devices (ONT) [58]. This method has been effectively employed for molecular epidemiology of Zika and Ebola outbreaks [58, 78, 79]. Targeted NGS is crucial for influenza surveillance, virus typing, and mutation detection. For example, its use in lower respiratory virus detection in ICU patients has identified H1N1pdm09 variants and 222G/N/A gene mutations, aiding in monitoring the speed and direction of influenza mutations [80]. The global pandemic of the novel coronavirus in 2019 has also provided opportunities for the application and advancement of targeted NGS, making it a widely used and optimized tool for tracking mutations in the SARS-CoV-2 genome and detecting emerging notable variants. For instance, high-density amplicon sequencing of symptomatic SARS-CoV-2 cases in suburban US regions has revealed ongoing viral evolution, with 57% of strains carrying the spike D614G variant. This finding also identified D614G as a dominant and emergent SARS-CoV-2 isolate in the United States [59, 81, 82]. In recent years, several researchers have been devoted to optimizing and implementing NGS-based amplicon sequencing methods for influenza surveillance [58, 83, 84] and identifying new RT-PCR target sites within the influenza genome [85]. Molecular monitoring and diagnostic technologies

have experienced unprecedented rapid development and transformation since this period [24].

### Application of untargeted sequencing (mNGS)

Respiratory infection is a major area where mNGS has shown significant benefit [66, 86]. mNGS does not rely on prior knowledge of pathogen genomes, enabling unbiased sampling and the detection of multiple pathogens within a sample. This is particularly valuable for sequencing clinical respiratory specimens of newly emerging pathogens [87], rare pathogens, infections of unknown etiology, or mixed infections. Additionally, mNGS can provide auxiliary genomic information needed for evolutionary tracing [88], strain identification [89], and resistance prediction [24, 90–94]. Samples of mNGS in detecting infectious diseases includes clinical respiratory specimens, samples from animal origins, and environmental samples.

## Respiratory specimens

For detecting bacterial pathogens in respiratory specimens, mNGS could be utilized to quantify microbial abundance, distinguishing between normal respiratory microbiota and true pathogens [95]. In one study, postmortem lung tissue mNGS was performed on a cohort of pediatric pneumonia fatalities in the United Kingdom and detected pathogens deemed to have directly contributed to death in 64% of cases [96]. Also, BAL mNGS from stem cell transplant recipients revealed additional potential pathogens (streptococcal mites, propionic acid rods, and four viruses) with increased abundance in 37% of routine-negative cases [97]. Additionally, while phenotypic antimicrobial susceptibility testing is the gold standard for determining bacterial pathogen susceptibility or resistance to antibiotics, mNGS can still identify potential drug-resistant bacterial hosts by providing detection results of Antimicrobial Resistance Genes (AMR), offering molecular biological insights for clinical management strategies [98]. For instance, Stoesser et al. reported a 95% concordance between phenotypic and WGS-predicted susceptibility for 7 commonly used agents—amoxicillin, amoxicillin/clavulanic acid, ciprofloxacin, gentamicin, ceftriaxone, ceftazidime, and meropenem. This was achieved by analyzing 143 assembled genomes from E. coli and K. pneumoniae against a compiled database of acquired AMR sequences and mutations in the quinolone-resistance-determining regions of gyrA and parC [99].

Due to its unbiased sampling approach, mNGS aids in the detection of new or unexpected pathogens in viral infections. For instance, community-acquired pneumonia (CAP) is a leading cause of pediatric hospitalization. Identifying the pathogen fails in approximately 20% of cases, yet it is critical for optimal treatment and the prevention of hospital-acquired infections. In one study, RNA mNGS was applied to respiratory samples from pediatric patients with CAP of unknown etiology and control respiratory samples. In addition to known viruses such as measles virus and parainfluenza virus, the detection results included many viruses clinically unknown or present in the control group, such as Anellovirus, Bocavirus, Human Herpes Virus-6, and −7 [95, 100], which demonstrated the promise of direct-from-specimen mNGS to enable challenging infection diagnoses.

## Animal and environment specimens

Samples from animals or the environment can also be analyzed using the mNGS method, which is a highly meaningful public health surveillance method for early warning and prediction of the spread and dissemination of respiratory pathogens.

By conducting mNGS testing and characterization of animals such as important vector arthropods and significant hosts of zoonotic diseases, it helps in developing a global map of potential zoonotic pathogens. This facilitates understanding of the respiratory pathogen sphere (especially viruses such as coronaviruses, influenza viruses, and Ebola virus [24]) and enhances the capability to discover, diagnose, and treat pathogens that pose significant threats to public health and global health. The Global Virome Project [101] is a prime example, covering known, emerging, and novel respiratory viral pathogens detected in important animal hosts and transmission vectors such as bats [102], wild ducks [103], mosquitoes [104], ticks [105, 106], and others, enriching the repository of zoonotic pathogens.

Currently, extensive research has been conducted on sampling various indoor and outdoor environments to explore environmental microbiomes. These studies have particularly focused on respiratory pathogens, examining their transmission, phylogeny, and antibiotic resistance genes. Among them, the large-scale metagenomic atlas of healthcare environments was first proposed [107, 108]. Notably, the first large-scale metagenomic atlas of healthcare environments have been proposed. Understanding the microbial composition, colonization, transmission patterns, and their influencing factors in hospitals is crucial [109], as hospital-associated outbreaks of viral respiratory illnesses pose significant infection control challenges. Conventional pathogen testing lacks the sensitivity to detect all pathogens and cannot assess epidemiological linkage between cases. Two studies evaluated outbreaks of severe respiratory illness using mNGS on bronchoalveolar lavage (BAL) specimens and showed enhanced outbreak epidemiology and transmission mapping [110, 111]. Additionally, mNGS on nasopharyngeal specimens during rhinovirus outbreaks in

a hematopoietic stem cell transplant unit revealed multiple cocirculating rhinovirus types, providing precise transmission insights and refining infection control interventions [112]. Multiple studies have described the value of mNGS for hospital environmental surveillance and monitoring [113, 114]. With the exponential growth of urban populations, monitoring urban public spaces, especially public transportation systems, is crucial for public health. The first large-scale metagenomic monitoring of the New York City subway system from 2013 to 2014 enhanced our understanding of pathogens and AMR genes in public transportation [114, 115]. Subsequently, the international MetaSUB consortium expanded this research to over 3,700 samples from 58 cities, establishing a global reference map of microbial diversity and AMR genes in urban public spaces, playing a crucial role in public health surveillance and response to emerging events [116].

## Advantages and remaining challenges

### Advantages over Conventional Diagnostics

Firstly, mNGS does not require assumptions about respiratory infectious pathogens, which facilitates early, rapid, and accurate reporting of results, significantly shortening diagnostic time [117]. In acutely ill hospitalized patients, infections often go undiagnosed, leading to inadequate treatment, prolonged hospitalization, readmissions and increased morbidity and mortality [118]. These patients are frequently immunocompromised, making them vulnerable to diverse respiratory pathogens including viruses, bacteria, fungi, and parasites. Conventional culture is limited by prior antimicrobial exposure and fastidious organisms. Targeted molecular testing like PCR can miss rare or divergent respiratory pathogens and is restricted to small genomic regions. A hypothesis-free approach enabling detection of any respiratory tract microbe would transform diagnostic microbiology, which is significant especially against the backdrop of the COVID-19 pandemic that the epidemiology of respiratory viruses has been continuously evolving [119]. Conventional methods have limitations in breadth of pathogen detection, leaving clinicians unsure whether negative results exclude an untested infection. mNGS provides clinical clues for subsequent diagnosis and treatment, particularly in avoiding overuse of antibiotics for viral infections. The rapid reporting of mNGS results can also facilitate timely adjustments in clinical practice for respiratory infections [117]. Secondly, mNGS allows unbiased broad-spectrum identification of expected and unexpected respiratory pathogens, even novel organisms [120]. Thus mNGS could be used to detect some rare as well as unknown or novel infectious pathogens. In previous reports, it has detected Francisella tularensis [121], brucellosis [122], cysticercosis, Taenia solium [123], Angiostrongylus cantonensis in cerebrospinal fluid [124], and hepatic tuberculosis in blood [124]. Thirdly, studies have shown that mNGS can not only be used for respiratory pathogen identification but also for microbiome characterization, parallel analysis of human host responses, and detection of antibiotic resistance genes and virulence factors [117]. All of these have led to the rapid development of mNGS in immunocompromised cases, difficult-to-diagnose respiratory cases, and patients with immunodeficiency [117].

It can also be coupled with targeted sequencing of conserved regions like 16 S and ITS for classifying bacteria and fungi in respiratory samples [125, 126]. mNGS provides additional genomic information for tracing phylogeny [127], strain typing [89] and predicting antimicrobial resistance [14]. Quantification of reads enables determining relative abundance in polymicrobial respiratory infections [125]. Additionally, traditional culture methods may be influenced by antibiotic use [128, 129], however, due to the longer survival time of pathogen DNA in plasma [117], the impact of antibiotics on mNGS results is minimal, thus expanding the applicability of mNGS methods in diagnosing respiratory infections.

### Remaining challenges

While great strides have been made in applying mNGS for clinical infectious disease diagnostics, barriers to full integration into routine clinical practice for respiratory infections remain. First and foremost is cost, with a typical mNGS test bill ranging from $500 to $1,000. As sequencers move closer to the true $100 human genome, costs should continue to decline [130]. Robotic automation of library preparation and sequencing workflow will drive efficiency and labor savings [131–133]. Multiplexing dozens of specimens into a single sequencing run and performing targeted enrichment steps are other ways to maximize information gain per dollar spent on sequencing for respiratory pathogens. Cloud computing and storage eliminate large capital equipment costs for analysis servers.

A major limitation of unbiased mNGS is the predominance of human nucleic acids, comprising > 99% of reads and limiting analytical sensitivity for respiratory pathogen detection [8, 126, 134]. Targeted sequencing of conserved regions like 16 S rRNA specifically captures bacterial sequences amidst host background. Host depletion methods increase the relative proportion of microbial reads by removing abundant human RNA species through hybridization, RNase H or CRISPR-Cas9 cleavage [8, 135–137]. These are effective for RNA but not DNA libraries, as capturing the entire human genome is impractical. Alternatives

targeting physical differences between pathogen and host nucleic acids include saponin lysis of human cells followed by DNase digestion, or size fractionation to isolate cell-free microbial DNA/RNA [134, 138–140]. Another challenge is contamination from reagents, environment or normal flora, requiring extensive quality control through negative controls, reagent screening and workflow separation [20, 110, 141]. Familiarity with expected microbial profiles for each specimen type facilitates interpretation.

Standardization of protocols for sample processing, nucleic acid extraction, library preparation, sequencing, bioinformatics analysis, and reporting is needed and will remove complexity as barriers to wide adoption of mNGS in respiratory diagnostics. Reference sequence databases are still incomplete; novel microbes and variants will continue to be discovered with unbiased mNGS. Development of curated databases and computational tools will aid in biological interpretation of novel findings.

Turnaround time is not yet rapid enough for clinical utility in many situations. However, rapid library preparation methods requiring only minutes of hands-on time have been designed [77]. Portable nanopore sequencing devices provide data acquisition and analysis in hours on-site [78]. As these tools mature, they will help position mNGS as a viable first-line diagnostic tool. Molecular point-of-care tests fill a useful niche currently, but they provide limited information focused only on a narrow range of anticipated respiratory pathogens. As unbiased mNGS reaches equivalent speed at lower cost per target organism, it may ultimately supplant limited multiplex molecular panels.

## Conclusions

Next-generation sequencing (NGS) technology has transformed clinical microbiology, and is playing an increasingly crucial role in diagnosing respiratory infectious diseases and epidemiological investigations, demonstrating outstanding potential in pathogen detection applications. This narrative review focuses on the application status of NGS in global respiratory infectious disease diagnosis, highlighting its exceptional potential in respiratory pathogen detection. Conventional methods for routine respiratory infection diagnosis, such as culture, smear microscopy, immunological assays, image techniques and PCR, have been summarized for comparison. In contrast, NGS offers a comprehensive detection technology that surpasses traditional methods in sensitivity and specificity. Here a detailed overview of NGS is provided, including its evaluation, workflow, and advantages over traditional techniques in detecting respiratory pathogens. While limitations of its costs and throughput persist, ongoing advancements are addressing these challenges. As sequencing technology continues to mature, NGS is poised to become a cornerstone technique in the global diagnosis of respiratory infections, offering rapid, accurate, and comprehensive pathogen identification.

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## Declarations

**Competing interests** The authors declare no competing interests.

**Ethics & Inclusion** No.

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