# Clinical diagnostic value of targeted next-generation sequencing for infectious diseases (Review)

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**Abstract.** As sequencing technology transitions from research to clinical settings, due to technological maturity and cost reductions, metagenomic next-generation sequencing (mNGS) is increasingly used. This shift underscores the growing need for more cost-effective and universally accessible sequencing assays to improve patient care and public health. Therefore, targeted NGS (tNGS) is gaining prominence. tNGS involves enrichment of target pathogens in patient samples based on multiplex PCR amplification or probe capture with excellent sensitivity. It is increasingly used in clinical diagnostics due to its practicality and efficiency. The present review compares the principles of different enrichment methods. The high positivity rate of tNGS in the detection of pathogens was found in respiratory samples with specific instances. tNGS maintains high sensitivity (70.8-95.0%) in samples with low pathogen loads, including blood and cerebrospinal fluid. Furthermore, tNGS is effective in detecting drug-resistant strains of *Mycobacterium tuberculosis*, allowing identification of resistance genes and guiding clinical treatment decisions, which is difficult to achieve with mNGS. In the present review, the application of tNGS in clinical settings and its current limitations are assessed. The continued development of tNGS has the potential to refine diagnostic accuracy and treatment efficacy and improving infectious disease management. However, further research to overcome technical challenges such as workflow time and cost is required.

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**Contents**



## 1. Introduction

Infectious diseases remain one of the top ten causes of human morbidity and mortality worldwide (1). Pathogen identification from clinical samples is key to guide treatment and management strategies for patients with infections. Currently, a number of traditional diagnostic techniques are used for etiological diagnosis, including microbial culture, antigen and antibody testing and PCR of microbial DNA or RNA (2). However, in ~50% of infected patients there are difficulties in pathogen identification due to the wide variety of pathogens, low throughput of traditional clinical methods and time-consuming nature of microbial culture (3).

Assays using next-generation sequencing (NGS) technology have potential to improve diagnosis of infectious diseases. NGS allows for sequencing of multiple individual DNA or RNA molecules in parallel, generating millions to billions of reads per run. The common applications of NGS in diagnostic microbiology laboratories include metagenomics NGS (mNGS) and targeted NGS (tNGS; Fig. 1). mNGS approaches characterize all DNA or RNA in a sample to enable analysis of the entire microbiome, whereas tNGS approaches enrich specific genetic targets to identify specific pathogens or genes of interest. Given the success of the clinical exploration of mNGS, attention has been focused on making NGS more widely and economically available in the clinical setting. The use of tNGS as a solution for this is also being assessed. The present review aimed to elucidate the diagnostic value of tNGS in clinical application.

## 2. Clinical application of mNGS for pathogen identification

As a successful example of the clinical application of NGS methods, previous studies have reported the promise of mNGS

in clinical application (4-14). mNGS successfully diagnosed neuroleptospirosis in a 14-year-old, which put the application of mNGS in the diagnosis of infectious diseases into the public light (4). In 2019, Blauwkamp *et al* (5) described the commercial mNGS sequencing Karius test (Karius, Inc.) in a cohort of 350 patients with suspected sepsis. It presented sensitivity of 92.9% and a specificity of 62.7% compared with the composite reference standard including culture, serology and nucleic acid testing and clinical adjudication when testing the plasma specimens from this cohort (5). Studies have confirmed the diagnostic value of mNGS in a number of patient cohorts assessing complex and atypical pathogen infection, including meningitis and pneumonia (3,6-9). mNGS is a potential method for identifying emerging or rare pathogens. For example, RNA-based mNGS identified the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as the cause of severe pneumonia (10). mNGS could provide clues for identifying rare pathogens including *Chlamydia psittaci*, *Mycobacterium tuberculosi*s (MTB), anaerobe and fungus (11-13). Moreover, sensitivity of mNGS is relatively less affected by prior antibiotics therapy compared to methods such as clinical cultures and immunologic assays. A previous study reported the sensitivity of mNGS is significantly higher than that of culture (52.7 vs. 34.4%) when patients had received prior antibiotic therapy (12). This conclusion was supported by Zhang *et al* (14), who found a higher sensitivity rate (66.67%) of mNGS in empirically-treated patients.

However, with widespread clinical use, shortcomings of mNGS have also received attention. The first problem is over-representation of human DNA in sequence data. As samples contain large amounts of human nucleic acid and the human genome is notably larger than the microbial genome, 80-99% of raw NGS reads are derived from human DNA (7,15), which decreases sensitivity of assays for microbial detection, especially when these are present in low abundance. Techniques for depleting host nucleic acid include physical methods such as centrifugation and filtration (16,17), chemical methods, including differential cleavage with chemical reagents (18), and selective hybridization removal using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated systems 9 (19) (Table I). However, these methods introduce new problems. Partial pathogens will be lost by these methods (20) and the risk of exogenous background contamination increases due to the addition of reagents. The complexity of these issues increases the difficulty of host nucleic acid depletion. At present, there is no relatively achievable method (20).

The detection performance of mNGS can be affected by the characteristics of pathogens. Small-genome, low-abundance pathogens such as viruses have limited coverage. A study assessing mNGS virus detection reported that median viral genome coverage is ~2% (21,22). In addition, antimicrobial resistance genes and virulence genes are also short in length, again a challenge for mNGS detection (23). Furthermore, the application of mNGS in fungal detection is limited. Pathogens with cell walls including fungi and intracellular bacteria affect nucleic acid extraction efficiency, which reduces the microbial nucleic acid abundance in sample (6,9,15,24-27). Besides, the high cost of mNGS limits its broader adoption in clinical settings. In China, the average cost per mNGS test is ¥3,000 (~US$400), which is significantly more expensive than individual traditional pathogen tests. For instance, culture tests cost around ¥600-700, the *Cryptococcus* antigen test is ¥320, and both the *Aspergillus* serological test and T-cell immunospot test T.SPOT are priced at ~¥600 (12).

## 3. Clinical application of tNGS

tNGS is an NGS method that detects a predetermined range of target genes. tNGS has been widely applied in disease identification including tumor detection, whereas studies assessing its use in pathogen detection are limited. Compared with mNGS, tNGS extracts predefined genes through enrichment, which allows pathogens to be targeted for sequencing. Targeted approaches are used to enrich known pathogens, especially low-concentration pathogens and their virulence and/or antimicrobial resistance genes in a single sample. This can increase the detection sensitivity for microorganisms being targeted, although it limits the breadth of potential pathogens that can be identified. Target enrichment is key to ensure sufficient sequencing depth and coverage in the regions of interest in tNGS workflow for accurate pathogen identification. Compared with mNGS, tNGS can enrich microbial nucleic acid content by several 10-fold to several 1,000-fold (28).

*Target enrichment approaches of tNGS*. Multiplex PCR amplicon-based and hybrid capture-based methodologies are used for target enrichment in tNGS. The multiplex PCR amplicon-based method amplifies and enriches target fragments through multiple primers designed for target region fragments. Multiple target fragments are amplified at the same time for library construction and sequencing. Hybrid-capture targeted method uses probes which hybridize with target fragments to capture and enrich target regions. Hybrid capture-based assays use magnetic beads to purify captured fragments to remove non-specific hybridization and constructs a DNA library for sequencing. The main differences between the approaches are shown in Table II.

The advantages of the amplicon-based method are low cost and quick and simple operation. The amplicon-based method can detect pathogens with low concentration. A study assessing the corona virus disease 2019 (COVID-19) detection ability of these methods reported that amplicon- and hybrid probe-based methods generally have the same enrichment effect on target gene fragments, but the amplicon-based method has a better enrichment effect for lower concentration viruses $<1x10^4$ copies/ml or Ct>28.7 (29). Moreover, the specificity of the amplicon-based method is better according to the stringency of the primer binding to the target region. Therefore, this method more efficiently detects single nucleotide variations. However, the fact that it has undergone PCR amplification makes it difficult to quantify, which may result in poor performance in detecting copy number and structural variation (29).

There are limitations for amplicon-based assay in applications. Firstly, the amplicon-based method has limitations for the detection spectrum. To avoid dimerization between primers, only ~20,000 primers can be designed. Considering the specificity of pathogens, the melting temperature value and the secondary structure of primers, this number will be greatly

Table I. Strategies for removing host genes (16-19).

| Principle | Method | Advantages | Disadvantages |
|---|---|---|---|
| Size of cell | Filter or flow cytometry | Easy operation | Loss of genes from intracellular bacteria, fungi and parasites |
| Difficulty in disrupting the cell wall | Selective cleavage and enzymatic cleavage or chemical cross-linking | Easy operation and low cost | Loss of certain genes from pathogens whose cell walls are easily disrupted |
| Differences in nucleic acid modification | Methylation capture | Suitable for methylation cases | Difficult operation, high cost, poor effectiveness |

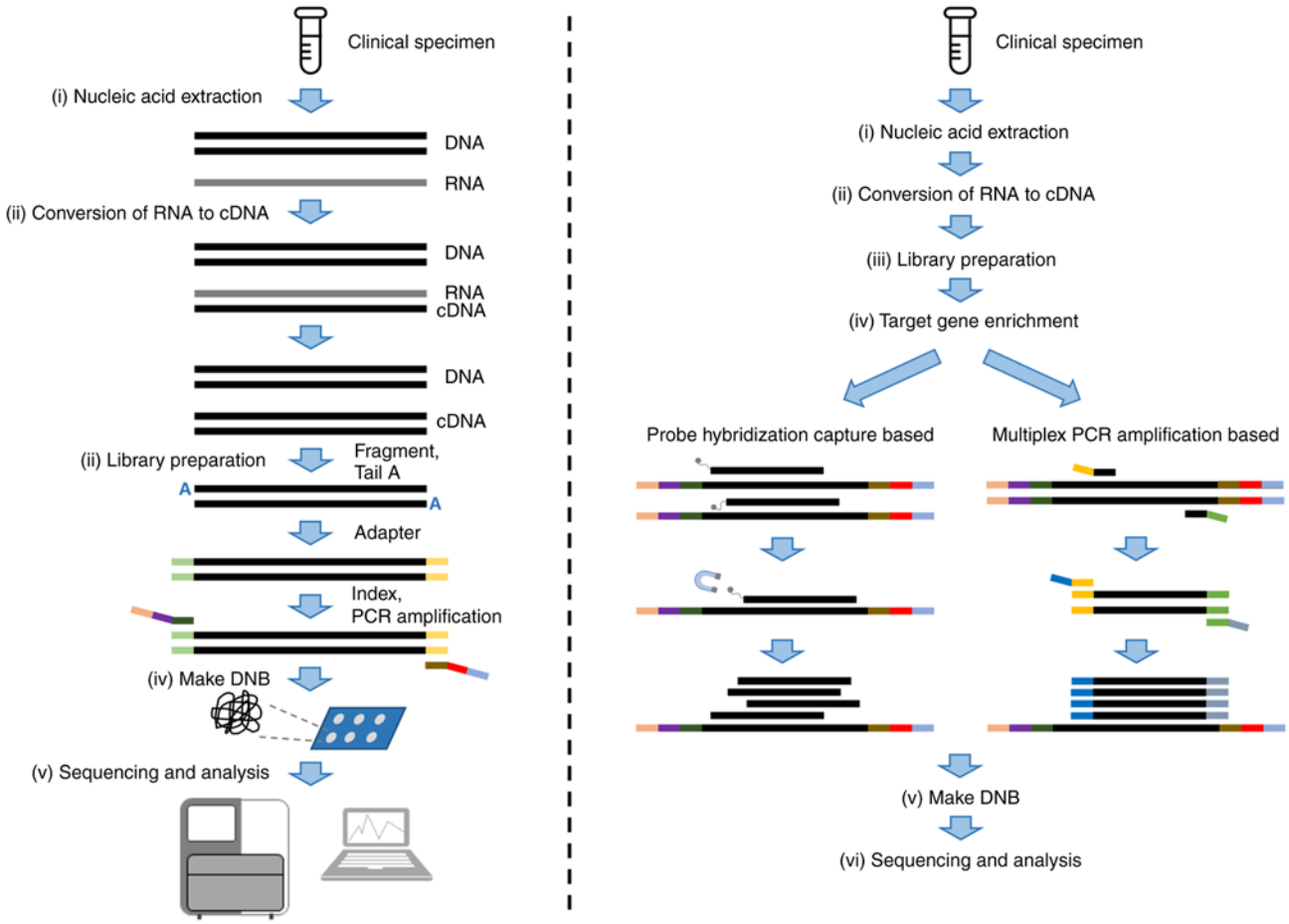


Figure 1. Workflow of metagenomic next-generation (left) sequencing and targeted next-generation sequencing (right). DNB, DNA ball; cDNA, complementary DNA.

reduced. This upper limit of the number of primers increases the difficulty of adjusting design of primers, which affects the flexibility of pathogen detection in the face of novel pathogens and different environments. Secondly, the amplicon-based method has a low tolerance for primer mismatch. This method requires almost 100% match between the primers and target sequence, rendering the amplification ineffective if mutations are present in the primer binding region. Thirdly, the amplicon-based method, used for sequencing SARS-CoV-2 genomes directly from clinical samples, has limitations in detecting minor alleles. Specifically, this method introduces biases that affect the accuracy of detecting the frequency of these minor alleles. Additionally, it has a significantly higher false-positive rate compared to the hybrid capture-based method (0.74 vs. 0.02%). This means that the amplicon-based method is less reliable and more prone to errors when identifying less prevalent genetic variations within the viral genome (29).

The hybrid capture-based method is able to detect a wider range of pathogens with more comprehensive genome coverage and more flexible pathogen detection design. The number of species detected by hybrid capture can be several 1,000. In the context of pandemics including COVID-19 and influenza A that require analysis of genome variation and virus monitoring

Table II. Multiplex PCR amplicon-based and hybrid capture-based methods.

| Factor | Multiplex PCR amplicon-based | Hybrid capture-based |
|---|---|---|
| Principle | Multiple primers to amplify and enrich target fragments | Design probes to hybridize with target fragments, capture and enrichment |
| Range | Dozens to hundreds | Thousands |
| Sample request | Low | High |
| Specificity | High | Low |
| Uniformity | Low | High |
| Flexibility | Poor | Good |
| Workflow time | Short (~12 h) | Long (~16 h) |
| Cost | Low | High |
| Operation | Simpler | Complex |

and traceability, the whole genome can be covered by the hybrid capture-based method. The hybrid capture-based method can combine new pathogen-specific probes without affecting performance. Furthermore, hybrid capture-based method has high tolerance for primer mismatch making it more suitable for use in samples with a high occurrence of mutations. Unlike the amplicon-based method, the hybrid capture-based method is able to amplify the target region with a matching accuracy of 70-80% (30). A genome sequencing study of hepatitis C virus (HCV) reported that the hybrid capture-based method can achieve the maximum capture efficiency of HCV at 80% probe matching (31). Furthermore, the hybrid capture-based method is less likely to capture identical nucleic acid fragments due to technical principles and therefore has a better uniformity. A previous study reported that the hybrid capture-based method has better uniformity and less bias than the amplicon-based method and may reflect the true content of pathogens in samples more objectively (29).

According to our experience, the hybrid capture-based method requires a higher initial concentration of pathogens and extracted DNA and cDNA need to be fragmented prior to probe hybridization capture. During fragmentation, loss of DNA and cDNA is possible. Furthermore, the probes may partially hybridize and capture non-specific fragments. IN addition, poor design of capture probes, suboptimal capture conditions, insufficient blocking of repetitive sequences in genomic DNA and improper ratio of genomic DNA to capture probe can affect capture specificity. Compared with the amplicon-based method, the hybrid capture-based method is more complicated, has a higher cost and longer workflow time. The time required for a typical probe hybridization capture process is ~12 h, which limits clinical application of this method. Rapid hybrid capture-based method can reduce the hybridization time to 2 h; however, time for the whole process of the hybrid capture-based tNGS is still longer than that of amplicon-based method at ~16 vs. ~12 h, respectively.

Enriching pathogen nucleic acid sequences being targeted can increase detection sensitivity (2). The virus identification ability between host depletion methods and target enrichment have been compared. Targeted capture-based approaches increase the nucleic acid of 90% viruses assessed, but host nucleic acid load was at times not reduced successfully (32). Zhao *et al* (22) used tNGS to enrich ribosomal RNA (rRNA) and reported that the quantity of microbial nucleic acid increases seven-fold. The detection results are consistent with the clinical findings for the identification of virus, fungus and antimicrobial resistance, demonstrating the accuracy of the detection method. Through enrichment, tNGS increases abundance and coverage of fastidious or low-abundance pathogens to improve reliability of detection results. In a study assessing fever in children using hybrid capture-based tNGS, the median increase in the reads of viruses as a percentage of all reads post-capture was 674-fold and the median genome coverage increased from 2.1 to 83.2% post-capture. This can also be applied for the analysis of an anrovirus, even if its sequences have up to 58% variation from the reference sequences used to select the capture probes (21).

The chemistry and software used in mNGS and capture-based tNGS and amplification-based tNGS vary notably. mNGS commonly uses reagents such as nucleotides and enzymes, including DNA polymerases (33). In addition to these, amplification-based tNGS requires primers specific to the targeted pathogens (34). Conversely, capture-based tNGS uses biotinylated oligonucleotide probes and streptavidin-coated magnetic beads instead of primers (35). Regarding bioinformatics analysis, sequence-based ultra-rapid+ pipeline is frequently used in mNGS for processing data, including the filtration of host sequences and identification of microbial sequences (36). The open-source platform IDseq is also widely applied in mNGS (37). Amplification-based tNGS often uses Primer3 for designing PCR primers and quantitative insights into microbial ecology for sequence analysis and microbial community profiling (38,39). Since capture-based tNGS is relatively new, researchers often develop custom pipelines in-house, combining steps for alignment, variant calling and annotation tailored to the captured targets (40). These techniques, along with associated costs, vary in terms of reagent and computational resource requirement. Amplification-based methods generally incur higher costs due to the necessity of specific primers and enzymes. By contrast, capture-based methods, while having high initial costs for probe design, benefit from lower ongoing operational costs. Notably, when considering the number of reads per workflow, the cost of capture-based tNGS is comparable to that of mNGS, at

US$150-160 per sample. Although capture-based tNGS requires notably less time for bioinformatics analysis compared with mNGS at 5 min vs. 1.5 h, it requires an additional 12 h for target enrichment (41).

*Diagnostic value of tNGS for clinical pathogen identification*

*Application of the preliminary tNGS in infectious disease*. In a study conducted in 2003, Gauduchon *et al* (42) reported an approach for the identification of pathogens in cases of infective endocarditis. This method involved application of broad-range eubacterial PCR amplification of the 16S rRNA gene, followed by sequencing of the patient heart valve material. The results demonstrated notable consistency between tNGS findings and histopathological evaluations with a high concordance rate of 93.1% (27/29) in positive samples and 92.9% (13/14) in negative samples (42). Studies assessed the use of tNGS methodology for pathogen identification in the clinical setting (43-55). The aforementioned studies suggested that tNGS could optimize antibiotic treatment decisions in a subset of patients, estimated at 10-20% of the cohort (42,46). In the broader context, tNGS may be a viable adjunct to traditional clinical detection methods, especially in scenarios where blood cultures yield negative results (44,45,47-50,52). However, relying solely on tNGS for clinical detection was not advisable, given its occasionally limited predictive power for negative samples (45).

A notable variant of preliminary tNGS integrates 16S rRNA gene PCR amplification with Sanger sequencing. A study using this method in clinical pathogen identification for infective endocarditis reported that sensitivity, specificity and positive predictive value and negative predictive value of tNGS were 92, 78, 78 and 92% respectively, compared with the culture methods, which reported values of 44, 100, 39 and 100%, respectively, demonstrating a significantly higher sensitivity (56). Further research on patients with infective endocarditis reported a similarly high positive ratio associated with this form of tNGS (50,57). Moreover, tNGS is used for pathogen identification in synovial fluid from patients with periprosthetic joint infections, where the sensitivity and specificity of tNGS are 69 and 100% respectively, which are lower compared with the culture method which had sensitivity and specificity values of 72 and 100%, respectively (58). Furthermore, a comparative study between these two forms of preliminary tNGS report that 16S rRNA gene PCR amplification followed by NGS exhibits higher accuracy compared with Sanger sequencing (59). Another study highlighted that after the sequencing method was changed from NGS to Sanger, based on 16S rRNA gene PCR amplification, notably increased the test positivity rate by 87% (60).

*tNGS in respiratory tract infections*. Amidst the challenges posed by the COVID-19 pandemic, tNGS has been used for infectious disease surveillance and genotyping (61,62). A comprehensive study including the surveillance data from six hospitals in Guangzhou (China) in 2022-2023 used a number of methodologies, including tNGS, to elucidate dynamics of the pandemic (61). The aforementioned study reported the Omicron BA.5.2 variant as the predominant strain in the region. Furthermore, ~49.8% of SARS-CoV-2 infections are concomitant with other pathogenic infections, underscoring the complexity of clinical management scenarios during the pandemic (61). Danilenko *et al* (63) applied tNGS for mutation analysis of the A(H1N1)pdm09 viruses, reporting an association between the *hemagglutinin* D222G/N mutation and mortality, thereby offering insight for effective disease prevention strategies. Moreover, Chao *et al* (64) reported the use of tNGS in pathogen identification in patients with acute lower respiratory tract infection. Benchmarked against the gold standard sputum culture, tNGS has a notable positive rate of 95.6% (64). tNGS can also be used in pediatric populations, with numerous studies reporting its clinical applicability (65-68). Lin *et al* (65) used respiratory pathogen identity/antimicrobial resistance enrichment sequencing (RPIP), a type of tNGS predicated on probe hybridization capture, for analysis of bronchoalveolar lavage fluid (BALF) samples from children with respiratory infections. RPIP demonstrated sensitivities and specificities of 84.4 and 97.7%, respectively, compared with culture-based gold standards (65). Moreover, another study reported the use of tNGS in identification of pneumonia in children through testing upper respiratory tract samples (66). Recent studies have reported the use of tNGS in identifying rare pathogens, including *Legionella pneumophila*, *Chlamydia psittaci*, *Tropheryma whipplei*, *Aspergillus fumigatus* and *Cryptococcus neoformans* (69-72).

A number of studies have reported the feasibility of tNGS in clinical application by comparing mNGS with tNGS (41,73). A study compared the performance of mNGS and tNGS in the detection of BALF specimens from patients with respiratory pathogen infection. tNGS demonstrated a similar performance to mNGS but the sequencing data were 1/3 of mNGS. The overall accuracy of tNGS workflow was 65.6%, which is similar to mNGS workflow at 67.1% (41). In another study of patients with lung infection, tNGS comprised 153 species and had a comparable detection rate to mNGS (82.17 vs. 86.51%, respectively). Detection rate of tNGS in bacteria, fungi and viruses was consistent with mNGS (73). These findings underscore the significance and practicality of tNGS in the clinical landscape, particularly in respiratory infectious disease.

*TNGS in bloodstream infection*. tNGS has been used to diagnose and manage bloodstream infection in hospitalized patients, owing to its high sensitivity, specificity and broad-spectrum pathogen detection capabilities. Studies (74,75) have reported the ability of tNGS to detect *P. falciparum*, a malaria-causing parasite (74). In Ghana, a tNGS approach using probes, has been used in the surveillance of malaria in pediatric populations (75). Furthermore, Deng *et al* (76) reported a metagenomic sequencing method using primer enrichment that enriches targeted RNA viral genomes. This methodology has improved viral diagnostics in blood, reporting a median ten-fold enrichment and a notable increase in genome coverage, which is particularly effective for hard-to-detect viruses such as Zika and Ebola (76). In a comprehensive study (77), a tNGS termed ultrasensitive single-genome sequencing (uSGS) was assessed. The aforementioned study used uSGS for the diagnosis of patients infected with HIV-1, reporting a >100-fold greater depth in HIV-1 RNA sequencing. This approach not only decreases PCR errors but also offers improved variant detection (77). Moreover, targeted amplification sequencing technologies including ampliseq detection system have demonstrated promising results in rapidly identifying pathogens directly

Table III. Application of tNGS in clinical pathogen identification.

| First author, year | Disease | Cohort size | Principle | Sensitivity, % | Specificity, % | PPV, % | NPV, % | (Refs.) |
|---|---|---|---|---|---|---|---|---|
| Maneg *et al*, 2016 | Infective endocarditis | 104 | 16S rRNA gene and NGS | 33.3 | 76.9 | 90.9 | 14.3 | (45) |
| Okuda *et al*, 2018 | Catheter-related infection | 156 | 16S rRNA gene and NGS | 80.0 | 76.9 | N/A | N/A | (54) |
| Flurin *et al*, 2021 | Periprosthetic joint infection | 105 | 16S rRNA gene and NGS | 85.0 | 98.0 | 98.0 | 89.0 | (55) |
| Miller *et al*, 2016 | Infective endocarditis | 68 | 16S rRNA gene and Sanger | 92.0 | 77.8 | 77.8 | 92.0 | (56) |
| Flurin *et al*, 2022 | Periprosthetic joint infection | 154 | 16S rRNA gene and Sanger | 69.0 | 100.0 | N/A | N/A | (58) |
| Chao *et al*, 2020 | Acute lower respiratory tract infection | 295 | Multiplex PCR amplification | 67.7-100.0 | 62.9-97.6 | N/A | N/A | (64) |
| Lin *et al*, 2023 | Pediatric respiratory tract infection | 47 | Probe hybridization capture | 84.4 | 97.7 | N/A | N/A | (65) |
| Li *et al*, 2021 | Pediatric severe community-acquired pneumonia | 47 | Multiplex PCR amplification | 87.1 | 100.0 | 100.0 | 64.2 | (67) |
| Gaston *et al*, 2022 | Lower respiratory tract infection | 177 | Probe hybridization capture | 45.9 | 85.7 | 76.7% | 60.7% | (41) |
| Deng *et al*, 2020 | Viral bloodstream infection | 39 | Multiplex PCR amplification | 95.0 | 94.8 | 92.7% | 96.5% | (76) |
| Gao *et al*, 2021 | Meningitis | 38 | Multiplex PCR amplification | 70.8 | 64.3 | 77.3% | 56.3 | (82) |
| Li *et al*, 2023 | Pediatric intracranial infection | 35 | Multiplex PCR amplification | 81.8 | 76.9 | N/A | N/A | (84) |

PPV, positive predictive value; NPV, negative predictive value; tNGS, targeted next generation sequencing; rRNA, ribosomal RNA.

Table IV. Performance of tNGS in MTB drug resistance testing.

| First author, year | Assay | Panel | Sensitivity, % | Specificity, % | Standard | (Refs.) |
|---|---|---|---|---|---|---|
| Jouet *et al*, 2021 | Deeplex Myc-TB | 13 drugs | 95.3 | 97.4 | WGS | (89) |
| Mansoor *et al*, 2023 | Deeplex Myc-TB | 13 drugs | 33.3-100.0 | 56.6-100.0 | pDST | (92) |
| Kambli *et al*, 2021 | Deeplex Myc-TB | 13 drugs | 83.5 | 100.0 | pDST | (94) |
| Wu *et al*, 2023 | TB tNGS | 4 drugs | 75.5 | 82.1 | pDST | (95) |
| Colman *et al*, 2016 | Next Gen-RDST | 7 drugs | 42.9-97.6 | 93.9-100.0 | pDST | (96) |
| Song *et al*, 2022 | tNGS | 9 drugs | 23.5-96.0 | 94.5.0 | pDST | (102) |

pDST, phenotypic drug susceptibility testing; tNGS, targeted next generation sequencing; MTB, *Mycobacterium tuberculosis*; WGS, whole-genome sequencing; RDST, rapid drug susceptibility test.

from blood samples, with a detection accuracy >92.81% in both spiked samples and clinical specimens (78). These results underscore the role of tNGS in improving diagnostic precision and guiding effective treatment strategies in clinical bloodstream infections.

*TNGS in central nervous system infections*. Recent case studies (79-81) robustly underscore the efficacy of tNGS in diagnosing central nervous system infection. For example, Jiang *et al* (79) reported a rare case of *Listeria monocytogenes* meningitis where tNGS successfully identified the pathogen and monitored the infection following antibiotic treatment, thereby demonstrating sensitivity in rare cases. Similarly, another study (80) demonstrated the use of tNGS with multiplex PCR amplification in the detection of herpes simplex virus type 1 in a patient with encephalitis. This supports the potential use of these methods in diagnosis and clinical surveillance of complex cases (80). Furthermore, Yang *et al* (81) reported a case of central nervous system aspergillosis identified by tNGS, which was initially misdiagnosed as *Toxoplasma gondii* encephalitis, demonstrating the effectiveness of tNGS for complex diagnoses.

In a broader clinical context, a study of patients with meningitis reported that tNGS exhibits improved sensitivity compared with mNGS at 70.8 vs. 41.7%, respectively. tNGS has improved diagnostic speed and cost-effectiveness. However, mNGS has specificity at 78.6 vs. 64.3%, respectively. These findings suggest that tNGS could be more suitable in certain clinical settings, such as when pathogens cannot be detected by conventional methods (82).

McGill *et al* (83) used virus capture-based sequencing for vertebrate viruses, another tNGS method based on probe hybridization capture, to detect unexpected viruses in cases of adult meningitis. The aforementioned study reported the potential of such technologies in identifying atypical viral agents and decreasing unnecessary antimicrobial use by guiding precision treatment (83). Notably, a study reported that in pediatric neurosurgery, tNGS demonstrates a higher sensitivity for diagnosing central nervous system infections (81.8% compared with 13.6% in traditional cerebrospinal fluid cultures and smears), demonstrating the diagnostic ability of tNGS techniques (84). The performance of tNGS pathogen identification in clinical studies is detailed in Table III.

*Robust detection of antimicrobial resistance*. tNGS identifies antimicrobial resistance genes by enriching target regions. In a study using RPIP for detection of pathogens and antimicrobial resistance genes in 201 respiratory tract samples, 53.8% of antimicrobial resistance genes reported by RPIP were consistent with clinical drug susceptibility testing (41). Nevertheless, the antimicrobial resistance effect of the same gene in different microorganisms is unequal. In the aforementioned study, the $bla_{OXA}$ genes encoding carbapenemases were detected in multiple *Pseudomonas aeruginosa* isolates; however, these were demonstrated to be susceptible to carbapenems. Therefore, considering the complexity of drug resistance genes and current databases requiring supplementation, it is necessary to analyze resistance genes in combination with pathogenic strains in single test. Lin *et al* (65) evaluated the efficacy of RPIP in pediatric patients with respiratory infections. The aforementioned study included 25 patients with confirmed *Streptococcus pneumoniae* infection who were tested for a spectrum of antimicrobial resistance genes. A total of 58 antimicrobial resistance genes associated with resistance to tetracycline, macrolide-lincosamide-streptogramin, β-lactams, sulfonamides and aminoglycosides, were identified in 19 patients. Compared with the results from clinical drug susceptibility testing, the concordance rates for erythromycin, tetracycline, penicillin and sulfonamides resistance are 89.5, 79.0, 36.8 and 42.1%, respectively (65). Application of tNGS for antimicrobial resistance gene detection has been extensively documented in an array of infectious diseases, including bloodstream infections, localized infection, infective endocarditis, urinary tract infections, malaria and leprosy (57,74,75,77,78,85-88).

*Application of tNGS in MTB*. In TB infections, the main challenge lies in the accessibility of efficient pathogen identification and comprehensive drug susceptibility testing. The World Health Organization recommended the Deeplex Myc-TB assay, a tNGS using multiplex PCR amplification (89-94). This assay has efficacy in prediction of resistance profiles against anti-tuberculous drugs (89-94). A study reported the high predictive accuracy of Deeplex Myc-TB with sensitivity of 95.3% and specificity of 97.4% in determining resistance to both first and second-line anti-TB medications. When used in the analysis of sputum samples, Deeplex Myc-TB parallels, and in some cases exceeds, performance of other advanced whole-genome sequencing methodologies (89). Sibandze *et al* (93) reported on the use of Deeplex Myc-TB with stool samples; this is an advancement for patient populations that are unable to provide sputum samples.

A number of other tNGS platforms have been used in the clinical application of TB testing. Predominantly, these platforms are used in the assessment of respiratory tract specimens (Table IV). For example, Wu *et al* (95) reported the efficacy of a multiplex PCR-based tNGS approach in the detection of MTB and its resistance to first-line drugs directly from BALF, demonstrating commensurate performance with traditional diagnostic methodologies, while assessing a broader range of resistance genes. Likewise, Colman *et al* (96) reported rapid and efficient characterization of drug resistant TB directly from sputum samples using amplicon sequencing. This method demonstrates a high concordance with phenotypic drug susceptibility testing and detects low abundance resistant variants (96). The effectiveness of tNGS in sputum samples detection has been validated by numerous subsequent studies (97-99). Extending beyond respiratory samples, tNGS has applicability in other types of clinical specimen. A study used tNGS for the detection of MTB in spinal tissues, demonstrating higher sensitivity compared with traditional culture methods at 100.00 vs. 18.75%, respectively. This approach successfully identifies antimicrobial resistance genes and mutations associated with drug resistance and suggests potential use in guiding personalized treatment strategies (100). A study assessing lung tissue samples from patients with TB reported the effectiveness of tNGS in detecting antimicrobial resistance (101). Furthermore, Song *et al* (102) reported the application of amplicon-based tNGS for diagnosing drug resistant TB using formalin-fixed and paraffin-embedded (FFPE) tissues. The aforementioned study reported that the detection sensitivity of tNGS for first-line drugs like rifampicin and isoniazid is high at 96 and 94%, respectively. The aforementioned study demonstrated the feasibility of using tNGS for FFPE samples, which can be challenging due to DNA degradation and reported advantages in terms of cost-efficiency, customizable for specific pathogen detection and reduced testing durations (102).

*Simplified interpretation and low cost of tNGS*. It has been reported that results obtained by tNGS are more concise than those reported by mNGS (41). In a comparative study of mNGS and tNGS, the number of analytes for mNGS workflow was 4-24 per sample, while one or two analytes were identified by tNGS (41). These results were compared with a comprehensive mNGS database, which covers tens of thousands of microorganisms, the tNGS database includes information on dozens to hundreds of target pathogens, with more in-depth and nuanced differentiation of subspecies and subtypes. In bioinformatics analysis workflows, 96.8% of microorganisms detected by mNGS are interpreted as non-pathogenic microorganism, while 75.2% microorganisms detected by tNGS are interpreted as non-pathogenic microorganisms (41). Moreover, refining the design of a database for tNGS is complex but necessary. A good design of the database can ensure accurate identification of pathogens. In a comparative study of tNGS and mNGS, *Pneumocystis carinii* was detected by both methods, but not reported, as it was considered a non-pathogenic microorganism for humans, and *Cryptococcus neoformans* was incorrectly reported as *Cryptococcus gattii* by tNGS (103).

Where mNGS sequences all nucleic acids present in a sample, tNGS only detects pathogens within the designed panel, resulting in a more stable performance and lower cost of sequencing at 1/3-1/2 of the cost of mNGS. Considering of the simplified interpretation and low cost, tNGS testing may be suitable for use in hospital microbiology laboratories. Overall, tNGS is more economical and may be more suitable for clinical application.

## 4. Prospects of tNGS

tNGS represents an advancement in infectious disease diagnostics, particularly in its capacity to identify a range of common pathogens via meticulously designed panels. This technology increases the reliability of detection results through its targeted amplification approach. tNGS may bridge the diagnostic gap between conventional tests and mNGS. tNGS has attracted attention of the clinical testing community. However, operational efficiency and applicability of tNGS are dependent on a number of factors, including the need to define the sample type, target pathogen range, positive judgment value and supporting database.

tNGS may not operate in isolation but in conjunction with conventional testing methods. This approach may contribute to effective and precise clinical diagnosis. By combining the universality of conventional tests with high specificity and sensitivity of tNGS, clinicians may be better equipped to make more accurate diagnoses. This integrated diagnostic strategy may improve patient outcomes by enabling timely and appropriate therapeutic interventions.

In summary, tNGS may serve a role in infectious disease diagnostics. By addressing limitations of current testing methodologies, tNGS may provide a more refined, accurate and comprehensive approach to pathogen detection. Its continued development and integration into clinical practice may impact infectious disease diagnosis and management.

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## Availability of data and materials

Not applicable.

## Authors' contributions

QYC and JY supervised the study and wrote the manuscript. YWL, YL, CLY, YJS and JD analyzed data. DJG and HL wrote the manuscript. YC and YCX reviewed the manuscript. All authors have read and approved the manuscript. Data authentication is not applicable.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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