

Enhancing lower respiratory tract infection diagnosis: implementation and clinical assessment of multiplex PCR-based and hybrid capture-based targeted next-generation sequencing



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Summary

Background Shotgun metagenomic next-generation sequencing (mNGS) is widely used to detect pathogens in bronchoalveolar lavage fluid (BALF). However, mNGS is complex and expensive. This study explored the feasibility of targeted next-generation sequencing (tNGS) in distinguishing lower respiratory tract infections in clinical practice.

Methods We used 229 retrospective BALF samples to establish thresholds and diagnostic values in a prospective cohort of 251 patients. After target pathogen selection, primer and probe design, optimization experiments, and bioinformatics analysis, multiplex PCR-based tNGS (mp-tNGS) and hybrid capture-based tNGS (hc-tNGS), targeting 198 and 3060 pathogens (DNA and RNA co-detection workflow) were established and performed.

Findings mp-tNGS and hc-tNGS took 10.3 and 16 h, respectively, with low sequencing data sizes of 0.1 M and 1 M reads, and test costs reduced to a quarter and half of mNGS. The LoDs of mp-tNGS and hc-tNGS were 50–450 CFU/mL. mp-tNGS and hc-tNGS were highly accurate, with 86.5% and 87.3% (vs. 85.5% for mNGS) sensitivities and 90.0% and 88.0% (vs. 92.1% for mNGS) specificities. tNGS detection rates for casual pathogens were 84.3% and 89.5% (vs. 88.5% for mNGS), significantly higher than conventional microbiological tests ($P < 0.001$). In seven samples, tNGS detected *Pneumocystis jirovecii*, a fungus not detected by mNGS. Whereas mNGS detected six samples with filamentous fungi (*Rhizopus oryzae*, *Aureobasidium pullulans*, *Aspergillus niger* complex, etc.) which missed by tNGS. The anaerobic bacteria as pathogen in eight samples was failed to detect by mp-tNGS.

Interpretation tNGS may offer a new, broad-spectrum, rapid, accurate and cost-effective approach to diagnosing respiratory infections.

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Keywords: Metagenomic next-generation sequencing; Targeted next-generation sequencing; Pathogen; Bronchoalveolar lavage fluid; Pneumonia

Introduction

Lower respiratory tract infections (LRTIs) are a leading cause of morbidity and mortality worldwide, with a great medical burden.^{1,2} A wide range of pathogens, such as bacteria, viruses, and fungi, can lead to LRTIs with indistinguishable clinical presentations. Rapid and accurate detection of pathogens results in broad-spectrum

antibiotic reduction and promotion of patient recovery.³ However, conventional diagnostic methods have shortcomings regarding culture difficulties and long turn-around times (TAT) and require prior assumptions regarding the types of pathogens.³

With the development of next-generation sequencing (NGS) technology and the increase in its clinical use,

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Research in context

Evidence before this study

Popular use of shotgun metagenomic next-generation sequencing (mNGS) in pathogen identification and the technical advance of enrichment strategy have laid the foundation for the trending application of targeted NGS (tNGS) in respiratory tract infection. Currently, mature and commercial tNGS products usually employ two enrichment strategies, multiplex PCR and hybrid-capture. A concern is rising about what are the differences among mNGS, multiplex PCR-based tNGS (mp-tNGS), and hybrid capture-based tNGS (hc-tNGS) in routine practice. To the best of our knowledge, there are currently no head-to-head comparisons on the methodological elaboration and analytical performance of these methods, nor clinical studies with large prospective samples. However, the extent to which these new tNGS methods contribute to clinical decision-making, their strengths in pathogen detection, and the similarities and differences in their implementation in clinical practice remain important questions.

Added value of this study

In this study, mp-tNGS and hc-tNGS methods were established. Analytical performance (limit of detection, linearity, interference, and precision) was evaluated. Notably, it was found that the main interfering factors of the two tNGS assays were different in the interference experiment.

229 retrospective and 251 prospective bronchoalveolar lavage fluid (BALF) samples were involved in the clinical evaluation. Compared with the composite reference standard, the accuracy of both tNGS methodologies was fully validated. The detection rates for casual pathogens of the three NGS methods were all significantly higher than that of conventional microbiological tests ($P < 0.001$). Finally, the similarities, differences, advantages, and limitations of the three NGS methods were summarized.

Implications of all the available evidence

Our data demonstrated that tNGS is broad-spectrum, rapid, accurate and cost-effective in lower respiratory tract infection. Compared with mNGS, tNGS shows low sensitivity on *Mucorales*, non-tuberculous mycobacteria and anaerobic bacteria in patients with aspiration pneumonia (mp-tNGS), and strong ability on detecting *Pneumocystis jirovecii*. The results obtained from the data of analytical performance and clinical evaluation would enhance our comprehensive understanding of tNGS, which includes target selection, primer or probe design, optimization of experimental conditions, performance in prospective samples and clinical scenarios for tNGS. These results suggest that tNGS has large clinical application prospects in the field of infections in the future.

NGS has become an important method for diagnosing genetic diseases and cancer. Historically, the use of NGS for infection diagnosis can be traced back to the 2010s when metagenomic NGS (mNGS) was initially utilized for virus detection.⁴ Recent studies have shown that NGS technology has great potential for diagnosing clinically relevant microorganisms.^{5,6} Its possibility to assess all microorganisms in one test and fast response leads to a wide range of applications nowadays.^{7–9} However, several barriers, such as a high human host nucleic acids background, colonization discrimination, and high costs, must be overcome.³

In this context, broad-spectrum targeted NGS (tNGS) has emerged as a cost-efficient alternative to mNGS. Technically, the methodology of tNGS, involving the use of PCR or hybrid capture to enrich the targets before sequencing, has not significantly changed since the 2010s.¹⁰ Nevertheless, the target spectrum of tNGS has expanded from singleplex assays (e.g., 16S rRNA, 18S rRNA, ITS amplicon sequencing) or low-multiplex to high-multiplex, driven by the need to overcome the challenges of accommodating hundreds to thousands of primers in a single assay system. This advancement has enabled tNGS to surpass the limitations observed when using singleplex targets. Consequently, the groundwork laid by mNGS, singleplex amplicon sequencing, and the subsequent technical progress have laid the foundation

for the rise of multiplex PCR-tNGS (mp-tNGS) and hybrid capture-based tNGS (hc-tNGS) as the prominent trends in sequencing technology.

Some reports have demonstrated the clinical application of tNGS^{11–13} in different types of infections. The deployment of mNGS and tNGS for pathogen identification and their diagnostic value have been demonstrated in previous studies.^{6,11–13} The initial findings suggest that all three methods could assist in clinical decision-making.^{6,11–13} However, the differences in their clinical applications and suitability in different scenarios remain unclear. Studies directly comparing these methods in the same patient group are lacking. The extent to which these new tNGS methods' strengths in pathogen detection, and the similarities and differences in their implementation in clinical practice remain important questions. Therefore, this study aimed to explore the feasibility of tNGS in distinguishing lower respiratory tract infections in clinical practice.

Methods

Ethics approval and consent to participate

This study was approved by the Research Ethics Board of the Peking University People's Hospital (ID: 2023PHB078). All procedures involving human participants performed in this study were in accordance with

the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consents were obtained from all the patients enrolled in the study. This study was taken a trial registration in Chinese Clinical Trial Registry (ChiCTR2300073837, study leader Hui Wang, registration date 2023-07-24).

Sample size

Sample size calculations were conducted before the study's initiation by PASS 2021, v21.0.3. Compared with the composite reference standard, the expected sensitivity of tNGS was 0.8, the confidence level was 0.95, and the confidence interval width was 0.2; 130 LRTI samples were calculated for sensitivity analysis. The expected specificity was 0.8, the confidence level was 0.95, and the confidence interval width was 0.2; 70 samples of non-LRTIs were calculated for specificity analysis.

Study design and participants

All bronchoalveolar lavage fluid (BALF) samples were obtained from patients undergoing routine mNGS tests at the Peking University People's Hospital (PKUPH). Residual samples were collected in sterile tubes as part of routine clinical care. The non-duplicate patients with sufficient sample volume (≥ 600 μ L) and complete clinical information were enrolled. Patients aged < 18 years with insufficient sample volume or tNGS failure were excluded. Cultures, molecular, and serological tests were performed in-house at the Department of Clinical Laboratory at PKUPH.

In this retrospective study, samples were collected between May 2022 and May 2023. Residual samples were stored at -80 $^{\circ}$ C until the time of extraction. The samples were randomly selected for storage. In this prospective study, a cohort of adults with suspected LRTIs who underwent bendable bronchoscopy and routine mNGS between July and September 2023 was included. The samples were stored at 4 $^{\circ}$ C and tested within 7 days of collection (Supplementary Fig. S1).

mp-tNGS workflow construction

The panel design is based on a comprehensive compilation of sources. A survey was conducted on various types of documents, including clinical expert consensus and literature/books on infections^{14–17} (Fig. 1a). The mp-tNGS panel covered 198 pathogen targets commonly encountered in clinical scenarios. A full list of the target species identified by the mp-tNGS panel is presented in Supplementary Table S1. A reference database was curated mainly from NCBI Refseq/nt.¹⁸ Redundant sequences with high similarity were removed from the database. Phage sequences and artificial sequences such as plasmids were filtered. Based on assembly level, submission source, sequencing method and annotation information, the database was cleaned and annotated to

remove low-quality sequences, improve accuracy and reduce bias. Alignment was performed using MUSCLE (v3.8.31)¹⁹ with default parameters to obtain interspecific SNP profiles, allowing phylogenetic relationships and downstream analysis to be computed. Based on the database integrated above, target loci capable of precise species and strain identification were selected for primer design. Target genes recommended by classical PCR methods in the literature were selected, followed by conserved and specific regions assessed by bioinformatics evaluation. The key points for primer design are as follows: 1. The GC content (guanine-cytosine content) of primers is set in the range from 40% to 60%. 2. The length of the primers is controlled between 18 bp and 26 bp. 3. The T_m value of the primers is designed at approximately 60 $^{\circ}$ C. 4. Avoid self-dimers, hairpins and cross-dimers. Primer sets for over 300-plex amplification were designed, and more primers (≥ 5) were designed for significant pathogens (e.g., *Mycobacterium tuberculosis* complex [MTBC]) and pathogens that need to be typed (e.g., SARS-CoV-2). A PCR process was developed and optimized to efficiently amplify the target signals with high sensitivity. The composition and concentrations of the primer sets are shown in Supplementary Table S2.

hc-tNGS workflow construction

hc-tNGS is a technology based on regular mNGS library construction, with its probe hybridization capture process specifically binding to microorganisms in order to enhance the detection of pathogenic microorganisms. Therefore, probe hybridization capture is the key development step of hc-tNGS. This project designed and synthesized a microbial capture probe pool containing millions of probes targeting species-specific genes, species-conserved genes, related drug resistance genes, and virulence genes of more than 3000 common and rare pathogenic microorganisms in the clinic. In the systematic study of probe hybridization, the optimal probe dosage between 0.1, 0.2, 0.3, and 0.4 fmol was verified by using simulated samples, and 0.3 fmol was found to have the best performance in this reaction system. The optimal temperatures for hybridization, capture, and washing are 60 $^{\circ}$ C, 65 $^{\circ}$ C, and 68 $^{\circ}$ C, respectively. Although a 12-h hybridization results in the best detection performance, the clinical requirement of shorter TAT in pathogenic detection urges us to apply 30 min of hybridization in this project. As the 30-min capture time has a minor effect on the final detection performance, considering the need to balance TAT and performance in clinical application, 30-min hybridization is used in this project (Supplementary Fig. S2).

mp-tNGS and hc-tNGS nucleic acid extraction

BALF samples with high viscosity were diluted 1:1 with 0.1 M dithiothreitol before nucleic acid extraction. The 600 μ L sample and 1.5 g glass bead were agitated

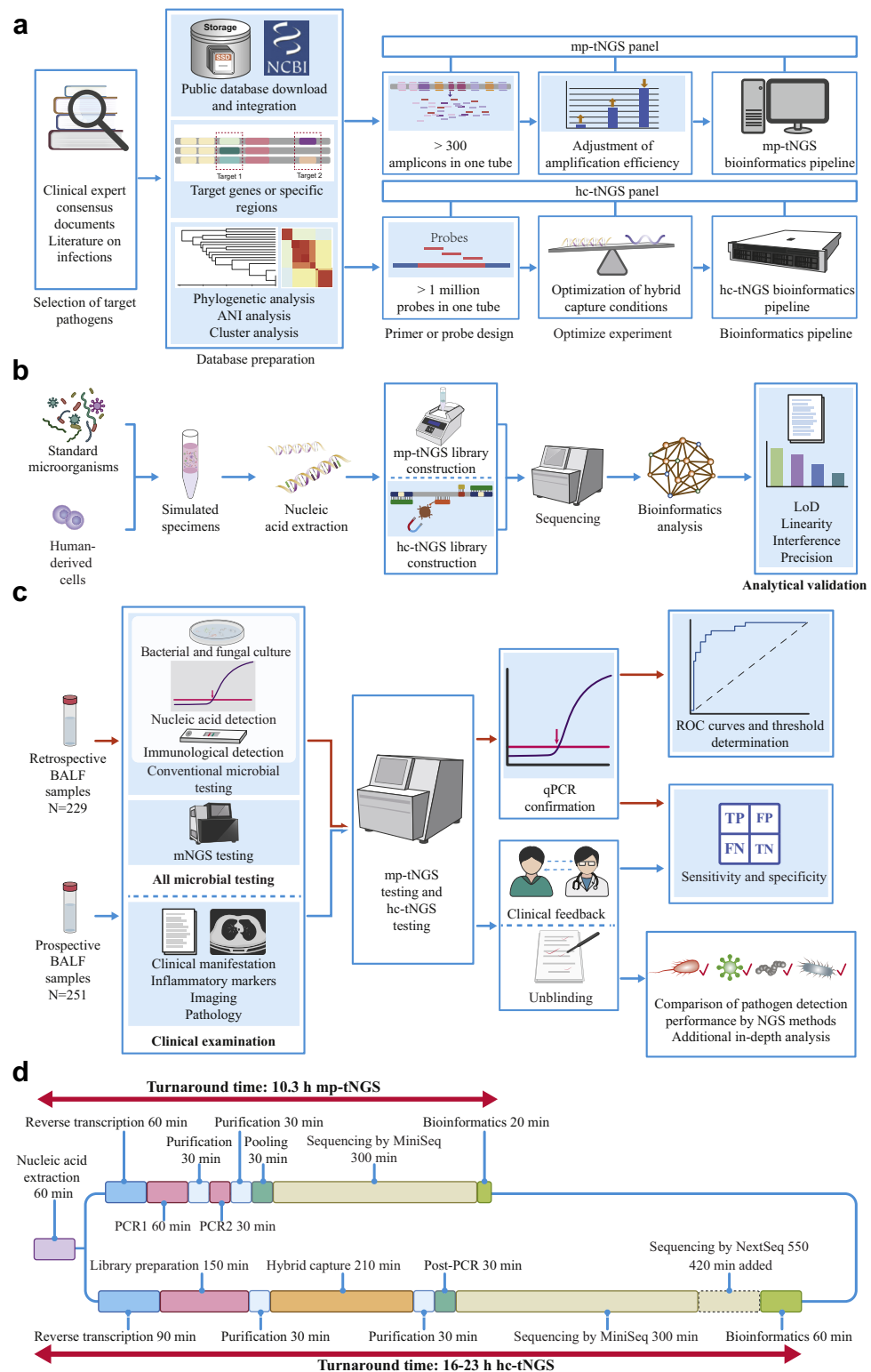


Fig. 1: Experimental design and analysis strategies for pathogen detection using NGS assays. (a) Design process of tNGS panels. (b) Workflow of tNGS assays for analytical performance. (c) Workflow of clinical analysis for pathogen diagnosis. Retrospective and prospective BALF samples were subjected to mp-tNGS and hc-tNGS testing, respectively, and the test results were compared with the gold standard (composite reference

vigorously at 4700 rpm for a total of 135 s by FastPrep-24™ 5G Instrument (MP Biomedical, CA, USA). The separated 0.25 mL sample was used to extract nucleic acids using the MagPure Pathogen DNA/RNA Extraction Kit (Magen Biotechnology, Guangzhou, China). Jurkat cells (c101-b; IGE Biotechnology, Guangzhou, China) were used as negative controls (NCs) to detect contamination, and Jurkat cells spiked with *Bacillus subtilis* (Guangdong Microbial Culture Collection Center, Guangzhou, China) were used as positive controls (PCs).

mp-tNGS library construction and bioinformatics pipelines

The target species covered by the mp-tNGS panel are listed in [Supplementary Table S1](#). The cDNA synthesis, multiplex PCR preamplification of target loci, and library preparation were performed using the RP100™ Respiratory Pathogen Multiplex Testing Kit (KingCreate, Guangzhou, China). Generated libraries were quantified using Equalbit DNA HS Assay Kit (Vazyme, Nanjing, Jiangsu, China) with Invitrogen™ Qubit™ 4.0 Fluorometer (Thermo Fisher, Waltham, MA, USA) to ensure all samples with library density ≥ 0.5 ng/ μ L or else subjected for library reconstruction. After library qualification, a single-end 100 bp sequencing was performed on the Illumina MiniSeq Platform using the MiniSeq Rapid Reagent Kit (100 cycles) (Illumina, CA, USA), with an average of 0.1 million sequencing reads for each sample.

Fastp v0.20.1²⁰ was employed for adapter trimming and low-quality reads filtering using default parameters, followed by alignment to the reference database (containing 683 bacteria, 372 viruses, and 349 fungi) using Bowtie2 v2.4.1²¹ in the “very-sensitive” mode. The numbers of reads per 100,000 sequencing reads (RPhK) were calculated at the species and genus levels. The thresholds were established based on the results of this retrospective study. The thresholds for viruses, bacteria, and fungi were 7, 15, and 11 RPhK, respectively.

hc-tNGS library construction and bioinformatics pipelines

Libraries were constructed using Reverse Transcription Kit (KingCreate, Guangzhou, China). Then, the cDNA was applied to Library Construction Kit (KingCreate, Guangzhou, China) to obtain DNA library. Eight

uniquely barcoded libraries were pooled to hybridize and capture by specific biotinylated probes for 0.5 h using the MetaCAP™ Pathogen Capture Metagenomic Assay Kit (KingCreate, Guangzhou, China). Libraries that passed quality control were quantified using the Equalbit DNA HS Assay Kit (Vazyme, Nanjing, Jiangsu, China), and sequencing was performed on the Illumina MiniSeq/Nextseq 550Dx platform using the MiniSeq Rapid Reagent Kit (100 cycles) or Nextseq 500/550 Mid Output Kit v2.5 (150 cycles) (Illumina, CA, USA) set to a 100-bp single-end with an average of 1 million reads per sample.

Clean reads were obtained by removing sequencing adapters, low-quality reads, excessive N bases (Reads containing more than five N bases were removed) or reads below 35 bp using fastp (version 0.23.1).²⁰ The remaining reads were aligned to the human reference (hg38) using Burrow-Wheeler Aligner (version 0.7.17-r1188), and human reads were filtered.²² The reads were compared to the classification reference database (containing 11,958 bacteria, 7373 viruses, 1714 fungi, and 343 parasites). The numbers of reads per one million sequencing reads (RPM) were calculated at the species and genus levels. The thresholds were established based on the results of this retrospective study. The thresholds for viruses, bacteria, and fungi were 6, 10, and 4 RPM, respectively.

Analytical performance

The limit of detection (LoD) was measured for ten representative pathogens (*Klebsiella pneumoniae*, *Legionella pneumophila*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus gattii*, Influenza A virus [FluA], Influenza B virus [FluB], and Herpes simplex virus 1 [HSV1]). The quantitative results of the viruses were the genome copy numbers measured by digital PCR (For FluA and FluB, provided and measured by National Institute of Metrology, China. For HSV1, provided by BDS Biological Technology, Guangzhou, China and measured by Forevergen Biosciences Centre, Guangzhou, China). The microorganisms were spiked into Jurkat cells (10^5 cells/mL) in a series of 3-fold dilutions (from 4050 CFU/mL to 50 CFU/mL) (copies/mL for the viruses). If a concentration of 50 CFU/mL was detectable, microorganism dilution continued until it was undetectable. Four replicates were performed for each concentration.

standard). The standard combines the evaluation results of all microbiological tests and clinical adjudication. Among them, all microbiological tests include conventional and mNGS testing. In this retrospective study (red arrows), confirmation by qPCR was added to improve pathogen detection information. The receiver operating characteristic (ROC) analysis was performed to establish tNGS thresholds and calculate the area under the curve (AUC). A prospective cohort study of 251 cases (blue arrows) was conducted in a double-blind study with other methods. Finally, after clinical feedback and unblinding, the performance of both tNGS methods was compared based on the composite reference standard and further in-depth analysis was conducted after unblinding. (d) Summary diagram of the experimental process time for tNGS assays. PCR1 stands for amplicon enrichment. PCR2 stands for Adapter-mediated PCR.

The LoD was defined as the dilution at which mp-tNGS or hc-tNGS detected the pathogen at levels above the threshold for simulated specimens (RPhK ≥ 15 with coverage of amplicons = 1 or RPhK ≥ 45 for mp-tNGS, and RPM ≥ 30 for hc-tNGS) in four replicates.

For linearity, the results (RPhK for mp-tNGS and RPM for hc-tNGS) of the same samples used to determine the LoD were plotted against the input concentration to perform a linear regression. The best-fit regression lines, linear equations, and R^2 values were added to the plotted values.

For precision, samples were spiked with seven representative organisms (*A. fumigatus*, *K. pneumoniae*, *S. pneumoniae*, *S. aureus*, *C. gattii*, FluA, and HSV1) at a concentration of $10 \times$ LoD. The intra-assay reproducibility detected five replicates of the same sample processed in parallel during the same run. Inter-assay reproducibility was analyzed using five replicates of the same sample across four independent tNGS runs.

Co-infection interference between high genomic-similarity microorganisms of the same genus, host interference, and competitive interference were analyzed. The performance of co-infection interference (high genomic-similarity microorganisms) was assessed using four cocktails of closely related species (*K. pneumoniae*/*K. oxytoca*, *S. pneumoniae*/*S. mitis*/*S. oralis*, *A. fumigatus*/*A. flavus*/*A. niger*, and Human adenovirus B3/Human adenovirus 5) of the same genus at different ratios (ratios of 1:3 and 3:1 in each species within a group, with a combination of equal proportions). Host interference was analyzed using a panel (1000 CFU/mL *K. pneumoniae*, *S. pneumoniae*, and *A. fumigatus* with 1000 copies/mL FluA) and different concentrations of Jurkat cells (10^4 , 10^5 , and 10^6 cells/mL). Competitive interference was performed on the strains (*K. pneumoniae*, *S. pneumoniae*, and *A. fumigatus*) at ratios of 1:1:1, 1:444:444, 444:444:1, and 444:1:444. Results are presented as the mean and standard deviation (SD) of three replicates.

mNGS nucleic acid extraction, library construction, and bioinformatics pipelines

The workflow was performed as previously reported.²³ DNA and RNA were extracted from the samples after host depletion using the PathoXtract® WYXM03202S universal pathogen enrichment extraction kit (WillingMed, Beijing, China). Human cells were lysed using a saponin buffer and centrifuged to remove the supernatant. DNA libraries were constructed using a Vazyme® TruePrep DNA Library Prep Kit V2 for Illumina (TD503; Vazyme, Nanjing, China). RNA libraries were constructed using the VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina (NR604; Vazyme, Nanjing, China). Library concentrations were quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher

Scientific, Waltham, MA, USA), and the libraries were mixed in equimolar amounts. A 75-bp single-end sequencing was performed using a NextSeq™ 550Dx instrument (Illumina, San Diego, CA, USA), with at least 10 million sequencing reads obtained for each sample.

The FASTQ format data obtained from sequencing were subjected to Trimmomatic v0.40²⁴ for high-quality sequencing data. Bowtie2 v2.4.3²¹ was used to compare the data with the human reference genome GRCh37 (hg19) to filter out human host sequences. The remaining sequences were aligned with a previously constructed reference database (including 24,000+ pathogens)²³ to identify the pathogens in the sample using Kraken2 v2.1.0.²⁵ The specific parameters used with Kraken were the reference database, which was the PlusPF database (downloaded on 9/12/2022) containing refseq archaea, bacteria, viral, fungi, and protozoa. The species-specific read number was normalized to RPM. The threshold criteria for determining pathogen positivity were established using the RPM ratio, which is the ratio between the RPM values of the sample and NC. For viruses, the threshold was detecting nonoverlapping reads from ≥ 3 distinct genomic regions and RPM ratio ≥ 3 . For bacteria and fungi, the threshold was RPM ratio of 10. Each batch's external PC (*B. subtilis*) and external NC (saline) run parallel to the clinical specimens throughout the workflow (nucleic acid extraction, library construction and sequencing).

Diagnostic assessment

The extra orthogonal testing, such as qPCR experiments for detecting EBV (Sansure Biotech, Changsha, China), CMV (Liferiver, Shanghai, China), HSV1, HSV2 (Liferiver, Shanghai, China), Flu A, Flu B, Rhinovirus, Human respiratory syncytial virus, Human mastadenovirus, *M. pneumoniae* (Sansure Biotech, Changsha, China), *S. aureus*, *S. pneumoniae*, *H. influenzae*, *K. pneumoniae*, *L. pneumophila*, *P. aeruginosa* (Sansure Biotech, Changsha, China) *Cryptococcus neoformans* capsular polysaccharide antigen assays (CrAg), and GeneXpert MTB/RIF assays (Xpert-MTB) were performed on samples, which had different results between mNGS and tNGS. Combined with laboratory and clinical data, the pathogen results were classified as definite, probable, possible, or unlikely according to the composite reference standard outlined in the Karius test²⁶ (Supplementary Fig. S3a). In separate cases, the categories of definite, probable, and possible pathogens were divided into causal and non-causal pathogens according to their clinical importance. Patients were considered negative for pathogens if causal pathogens were not detected. The clinical sensitivity and specificity of mp-tNGS, hc-tNGS, and mNGS were calculated and compared with those of

conventional microbiological testing (CMTs) (including culture, qPCR, serological tests, [1,3]- β -D-glucan and galactomannan tests, Xpert-MTB, and CrAg), and clinical adjudication.

Statistical analysis

The sample size was calculated using PASS software (v15.0; NCSS Corporation, USA). Student t-test, Mann–Whitney U test and the Chi-square test/correction for continuity of Chi-square test/Fisher's exact test were used to compare continuous and categorical data, respectively. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using SPSS software (v25.0; IBM Corporation, USA).

Role of funding source

The funders did not play any role in the study design, data collection, management, analysis, interpretation, review, approval of the manuscript, or the decision to submit the manuscript for publication.

Results

Analytical performance

An overview of the timeline for mp-tNGS and hc-tNGS is shown in Fig. 1d and a summary of the study design is shown in Fig. 1b and c. It takes approximately 1 h to perform multi-PCR to enrich the pathogens in mp-tNGS, which is shorter than the 3.5 h to hybrid capture in hc-tNGS. mp-tNGS had an overall sample-to-answer TAT of 10.3 h, whereas the TAT for hc-tNGS was 16–23 h.

The desired LoD of the tNGS panel development is 450 CFU/mL (copies/mL) for all target microorganisms, and the minimum sequencing data volumes for mp-tNGS and hc-tNGS is 24.2 K and 63.1 K, respectively, based on Bernoulli processes²⁷ (Supplementary Table S3). The LoD for bacterial detection was estimated to be 50–450 CFU/mL using both the mp-tNGS and hc-tNGS assays. For fungal detection, the LoD ranged from 50 to 150 CFU/mL in two ways. The viral LoD was 50–150 copies/mL in both assays. The LoDs for *S. aureus*, *A. fumigatus*, and HSV1 were slightly higher in mp-tNGS than hc-tNGS. The LoDs for *C. albicans* and *C. gattii* were slightly higher in hc-tNGS than mp-tNGS (Table 1 and Supplementary Fig. S4). For both methods, a strong linear correlation was observed between the microorganism titer and RPhK or RPM values. The mean R^2 was 0.83 (with SD of 0.14) and 0.91 (with SD of 0.07) (Supplementary Fig. S4).

The samples contained different concentrations of representative organisms to measure the precision and coefficient of variation (CV). The mp-tNGS and hc-tNGS assays showed high intra- and inter-assay reproducibility (100%). The CV for hc-tNGS was less than that for mp-tNGS in both intra- and inter-assays. For mp-tNGS, the mean intra- and inter-assay CVs were 28.15%

(SD = 10.50%) and 51.56% (SD = 17.96%), respectively. The mean CV for hc-tNGS was 14.30% (SD = 8.98%) in the intra-assay and 54.82% (SD = 16.25%) in the inter-assay (Table 1 and Supplementary Table S4).

Interference from high genomic-similarity microorganisms within the same genus was evaluated using mp-tNGS and hc-tNGS assays. The samples revealed accurate pathogens and were consistent with the mixed panel using mp-tNGS. In hc-tNGS, a few related species of the same genus (*Streptococcus* spp. and *Aspergillus* spp.) were incorrectly detected. However, no absolute correlation was observed between the proportions of species and the mixed ratio for either mp-tNGS or hc-tNGS (Table 1 and Fig. 2a) because of the unbalanced design of specific primers for different pathogens.

Host interference was evaluated using samples with different host burdens but the same organism burden. The results showed that mp-tNGS detection was not affected by host burden, whereas host burden had little impact on hc-tNGS detection (Table 1 and Fig. 2b). For competitive interference, both assays accurately detected spiked organisms with a further disparity in the spiked proportion (Table 1 and Fig. 2c). The detected mp-tNGS sequences had greater bias than hc-tNGS.

Study patients

A summary of fundamental information, the results of laboratory tests, and the discrepancies in pathogen detection is presented in Supplementary Table S5. In this retrospective study, 250 samples met the inclusion criteria, of which 21 were excluded because of the patient's underage or insufficient volume. Of the 229 patients, the final pulmonary diagnoses included respiratory infections (63.8%), tumors (6.6%), and pulmonary abscesses (1.7%). In this prospective study, 251 patients with suspected pneumonia were included after excluding one duplicate case. The final pulmonary diagnoses of the 251 patients included respiratory infections (68.1%), tumors (7.2%), and pulmonary abscesses (4.0%). No significant differences were observed in sex, age, in-hospital and underlying diseases, 30-day mortality, or laboratory examinations between the two groups. A higher proportion of patients were admitted to the intensive care unit in the prospective study (23.1% vs. 7.9%, $P < 0.001$, Chi-square test, Table 2).

Diagnostic performance of mp-tNGS and hc-tNGS

In this retrospective study, thresholds were established using the clinical results of 229 patients. For mp-tNGS, the thresholds for viruses, bacteria, and fungi were 7, 15, and 11 reads per 100,000 sequencing reads (RPhK), respectively. For hc-tNGS, the thresholds for viruses, bacteria, and fungi were 6, 10, and 4 reads per million (RPM), respectively. The two methods showed good diagnostic performance (area under the receiver operating characteristic [ROC AUC] ≥ 0.822 for all

Performance metric	Method/Pathogen type	Representative organism	Results	
			mp-tNGS assays	hc-tNGS assays
Limits of detection (LoD)	Bacteria, gram-negative	<i>Klebsiella pneumoniae</i> ATCC 10031	50 CFU/mL	50 CFU/mL
		<i>Legionella pneumophila</i> ATCC 33152	450 CFU/mL	450 CFU/mL
	Bacteria, gram-positive	<i>Streptococcus pneumoniae</i> ATCC 49619	150 CFU/mL	150 CFU/mL
		<i>Staphylococcus aureus</i> ATCC BAA-976	450 CFU/mL	150 CFU/mL
	Fungi	<i>Candida albicans</i> ATCC 14053	50 CFU/mL	150 CFU/mL
		<i>Aspergillus fumigatus</i> ATCC 96918	150 CFU/mL	50 CFU/mL
		<i>Cryptococcus gattii</i> ATCC 34877	50 CFU/mL	150 CFU/mL
	RNA Viruses	Influenza A (H1N1) pdm09 virus NIM-RM4054	150 copies/mL	150 copies/mL
		Influenza B virus (B/Victoria) NIM-RM4056	50 copies/mL	50 copies/mL
Precision	DNA Viruses	Herpes simplex virus 1 BDS-IQC-032	150 copies/mL	50 copies/mL
	Intra-assay consistency performance detecting five replicates of the same sample containing seven organisms at ratios of 10 × LoD processed in parallel on the same run	<i>Aspergillus fumigatus</i> / <i>Klebsiella pneumoniae</i> / <i>Streptococcus pneumoniae</i> / <i>Staphylococcus aureus</i> / <i>Cryptococcus gattii</i> /Influenza A virus/Herpes simplex virus 1	100% concordance	100% concordance
		Inter-assay consistency analyses five replicates of the same sample across five independent tNGS runs	100% concordance	100% concordance
Interference	Co-infection interference (high genomic-similarity microorganisms) performance was assessed using cocktails of close species in the same genus at different levels	<i>Klebsiella pneumoniae</i> / <i>Klebsiella oxytoca</i> / <i>Streptococcus pneumoniae</i> / <i>Streptococcus mitis</i> / <i>Streptococcus oralis</i> / <i>Aspergillus fumigatus</i> / <i>Aspergillus flavus</i> / <i>Aspergillus niger</i> /Human adenovirus B3/Human adenovirus 5	Not interfered by high genomic-similarity microorganisms	A little interfered by high genomic-similarity microorganisms
		<i>Klebsiella pneumoniae</i> / <i>Streptococcus pneumoniae</i> / <i>Aspergillus fumigatus</i> /Influenza A virus	Not interfered	Has little impact on the detection
	Competitive interference analyses on representative organisms at different ratios of four remote phylogenetic groups	<i>Klebsiella pneumoniae</i> / <i>Streptococcus pneumoniae</i> / <i>Aspergillus fumigatus</i>	Has a minimal impact on the detection	Little effect

Table 1: Performance characteristics of the mp-tNGS and hc-tNGS assays.

pathogens). Both mp-tNGS and hc-tNGS were better at detecting viruses (ROC AUC: 0.932 and 0.952, respectively) and bacteria (ROC AUC: 0.846 and 0.874, respectively) than fungi (ROC AUC: 0.833 and 0.802, respectively) (Fig. 3a). Compared to culture, the sensitivities of mp-tNGS and hc-tNGS were 94.7% (95% confidence interval [CI]: 82.3–99.4%) and 89.5% (95% CI: 75.2–97.1%), respectively, with low specificities of 57.8% (95% CI: 50.3–64.9%) and 56.0% (95% CI: 48.7–63.2%), respectively. The sensitivities of mp-tNGS and hc-tNGS were 86.2% (95% CI: 79.0–91.6%) and 82.7% (95% CI: 75.2–88.7%), respectively, when compared with the composite reference standard, while mp-tNGS (96.8% [95% CI: 91.0–99.3%]) and hc-tNGS (91.7% [95% CI: 84.2–96.3%]) showed higher specificities (Fig. 3c). For mNGS, the sensitivity and specificity were 88.4% (95% CI: 81.5–93.3%) and 90.4% (95% CI: 82.6–95.5%) compared with the composite reference standard (Supplementary Fig. S5).

Of 251 patients in the prospective study, 91 showed positive results in CMTs. Pathogens were detected in 129 cases by mp-tNGS or hc-tNGS (Fig. 3b). Compared

to culture, the sensitivities of mp-tNGS and hc-tNGS were 96.8% (95% CI: 83.3–99.9%) and 100.0% (95% CI: 88.8–100.0%), with the specificities of 47.2% (mp-tNGS [95% CI: 40.4–54.1%]) and 46.3% (hc-tNGS [95% CI: 39.6–53.2%]). Compared with the composite reference standard, the sensitivities of mp-tNGS and hc-tNGS were 86.5% (95% CI: 80.0–91.4%) and 87.3% (95% CI: 81.0–92.0%), respectively. Additionally, mp-tNGS (90.0% [95% CI: 81.9–95.3%]) and hc-tNGS (88.0% [95% CI: 79.6–93.9%]) showed lower specificities than those in the retrospective study (Fig. 3c). For mNGS, the sensitivity and specificity were 85.5% (95% CI: 79.1–90.6%) and 92.1% (95% CI: 84.5–96.8%) compared with the composite reference standard (Supplementary Fig. S5). The potential pathogens detected by mp-tNGS and hc-tNGS in prospective studies were shown in Supplementary Fig. S6, and both two tNGS methods had a good consistency.

Clinical evaluation of mp-tNGS, hc-tNGS, vs. mNGS

In this prospective study, 251 samples were used to evaluate the clinical diagnostic performance of

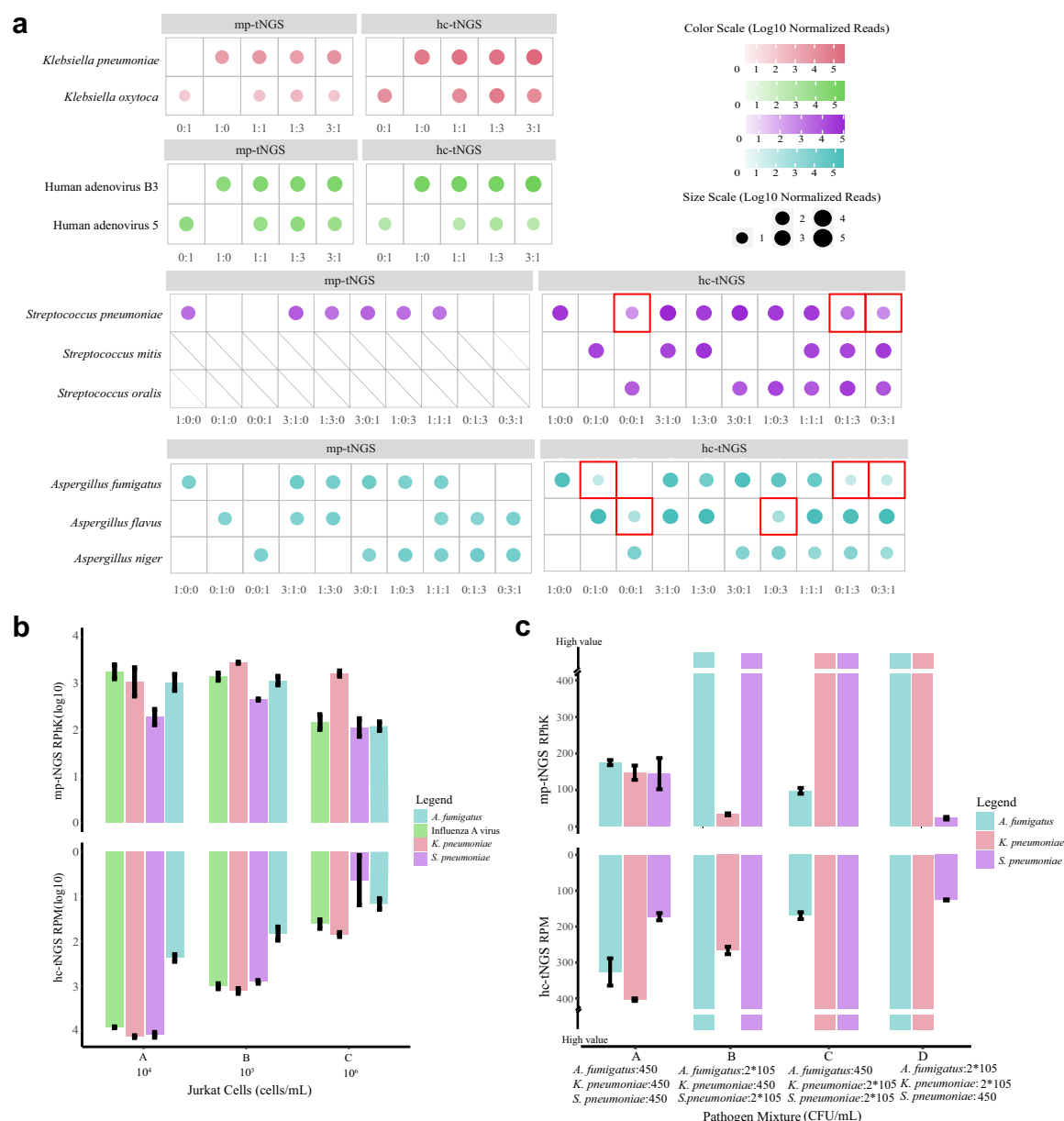


Fig. 2: Analysis of interference testing. (a) Interference testing for closely related species detection. Four groups of samples were selected for interference testing, including *Klebsiella* spp., Mastadenovirus, *Streptococcus mitis* group, and *Aspergillus* spp. The 1:3, 3:1, and 1:1 ratios were used as the experimental groups for pairwise cross-testing, with 0:1 and 1:0 ratios as the control reference. The larger the circle in the figure, the darker the color, indicating a higher number of normalized reads. The red box indicates the presence of non-specific sequences, and the diagonal line indicates that they are not within the detection range of mp-tNGS. (b) Human source sequence interference testing. A, B, and C groups used three concentration gradients of 10⁴, 10⁵, and 10⁶ cells/mL, respectively, with a concentration of 1000 copies/mL (CFU/mL) for the four pathogens. (c) Pathogenic competition interference testing. The test was divided into four groups: A, B, C, and D. Among them, the three pathogens in Group A were all 450 CFU/mL. B, C, and D alternately increased the two pathogens to 2 × 10⁵ CFU/mL.

mp-tNGS, hc-tNGS, and mNGS. Almost half of the pathogens (224/465) were detected using all three methods (Fig. 4a). A higher proportion of RNA viruses was detected in the pathogens co-detected by both mp-tNGS and hc-tNGS, while DNA viruses and bacteria accounted for a higher proportion of pathogens detected

by both hc-tNGS and mNGS (Fig. 4a). The normalized reads were higher in the causal pathogen group than in the non-causal pathogen group (Fig. 4b), which was more significant according to the composite reference standard (Supplementary Fig. S7). The lists of colonizing bacteria and other non-pathogenic

Characteristics	Retrospective study (n = 229)	Prospective study (n = 251)
Age (years)		
Median (IQR)	59 (44–68)	62 (50–69)
Range	21–85	21–91
Sex, n (%)		
Female	122 (53.28)	126 (50.20)
Male	107 (46.72)	125 (49.80)
In hospital, n (%)	222 (96.94)	234 (93.22)
In intensive care unit, n (%)	18 (7.86)	58 (23.11)
30-day mortality, n (%)	6 (2.62)	10 (3.98)
Immunocompromised, n (%)	72 (31.44)	85 (33.86)
Underlying conditions, n (%)		
Respiratory diseases	127 (55.46)	155 (61.75)
Hematologic tumors	35 (15.28)	48 (19.12)
Blood system diseases	13 (5.68)	18 (7.17)
Solid organ tumors	34 (14.85)	38 (15.14)
Autoimmune diseases	52 (22.71)	33 (13.15)
Metabolic diseases	105 (45.85)	123 (49.00)
Circulatory system diseases	82 (35.81)	93 (37.05)
Digestive system diseases	53 (23.14)	73 (29.08)
Nervous system diseases	20 (8.73)	23 (9.16)
Urinary system diseases	26 (11.35)	23 (9.16)
Mental diseases	7 (3.06)	7 (2.79)
WBC, median (IQR) ($\times 10^9/L$)	6.37 (4.9–8.33)	6.39 (4.76–8.64)
Range	0.1–27.24	0.3–39.95
Neutrophil ratio, median (IQR) (%)	67.2 (55.9–77)	67 (56.6–77.6)
Range	1.9–97.5	2–96.3
Lymphocyte ratio, median (IQR) (%)	20.8 (13.8–30.8)	22 (13.7–31.4)
Range	2.3–77.1	0.8–97.3
CRP, median (IQR) (mg/L)	5.1 (1–34.4)	10.5 (2.1–46)
PCT, median (IQR) (ng/mL)	0.05 (0.03–0.12)	0.06 (0.04–0.13)
Final pulmonary diagnosis, n (%)		
Respiratory infection	146 (63.76)	171 (68.13)
Tumor	15 (6.55)	18 (7.17)
Pulmonary abscess	4 (1.75)	10 (3.98)
Treatment modification based on mNGS results, n (%)	109 (47.60)	120 (47.81)

Abbreviation: IQR, interquartile range; WBC, whole blood count; CRP, C-reactive protein; PCT, procalcitonin; mNGS, metagenomic next-generation sequencing.

Table 2: Demographic and clinical characteristics of the cohort.

microorganisms for hc-tNGS and mNGS were shown in [Supplementary Tables S6 and S7](#).

Moreover, 251 samples were used to analyze which of the three methods could detect different types of causal pathogens ([Fig. 4c](#) and [Supplementary Table S8](#)). mNGS performed better against rare pathogens (such as *Rhizopus oryzae*, *Aureobasidium pullulans*, and *Aspergillus niger* complex), whereas tNGS detected more viruses and *Pneumocystis jirovecii*. Eighteen causal pathogens detected only by mNGS were nine bacteria (MTBC, *Mycobacterium avium* complex [MAC], *P. aeruginosa*, *K. pneumoniae*, and *S. maltophilia*, *Acinetobacter pittii*), two RNA viruses (SARS-CoV-2 and Human orthorubulavirus 4), and six filamentous fungi (*R. oryzae*,

A. pullulans, *A. flavus* complex, *A. fumigatus*, *Cladosporium sphaerospermum*, and *A. niger* complex), and one yeast (*Meyerozyma guilliermondii*). The 23 causal pathogens detected by both tNGS but missed by mNGS were nine fungi (*P. jirovecii*, *A. fumigatus*, and *Rhizopus microsporus*), three DNA viruses (EBV and HSV1), seven RNA viruses (SARS-CoV-2, Rhinovirus, and Respiratory syncytial virus B, Human coronavirus OC43, Human orthorubulavirus 4), and four bacteria (*S. pneumoniae*, *K. pneumoniae*, and *Mycobacterium abscessus* complex). Five out of seven RNA viruses remained unidentified by mNGS, possibly due to missing RNA workflow, which was not requested by the physicians. Seven of the nine missing detected fungi by mNGS were *P. jirovecii*. Twenty-one bacteria were detected by hc-tNGS and mNGS but missed by mp-tNGS, as 16 of the 21 in eight samples were anaerobic bacteria in patients with lung abscesses or aspiration pneumonia. Overall, for mp-tNGS, 9% of DNA pathogens were missed due to panel setting, and for mNGS, 18% of RNA pathogens were missed due to no RNA detection ([Supplementary Fig. S8](#)).

In 251 prospective patients, the proportion of causal pathogens that could be detected using each method was measured. Thus, the detection rates of causal pathogens were 84.3% (95% CI: 78.9–88.7%), 89.5% (95% CI: 85.0–93.0%), and 88.5% (95% CI: 83.8–92.2%) for mp-tNGS, hc-tNGS, and mNGS, respectively ([Fig. 4c](#) and [Supplementary Fig. S9](#)). Furthermore, the detection rates of three NGS methods were all significantly higher than those of CMTs ($P < 0.001$, Chi-square test/Fisher's exact test, [Supplementary Fig. S9](#)). The pathogens missed by CMTs but detected by NGS were shown in [Supplementary Table S9](#). The pathogen spectrum of tNGS could cover more than 95% (245/251 [mp-tNGS] and 249/251 [hc-tNGS]) of the cases ([Supplementary Table S5](#)).

To evaluate the detection performance of the five significant pathogens compared to traditional routine clinical tests, clinical judgment, mNGS, mp-tNGS, hc-tNGS, CrAg, Xpert-MTB, and galactomannan assays (GM) of all relevant cases in this study were integrated ([Fig. 5](#)). The sensitivity of *C. neoformans* Ag was significantly higher than that of mNGS and tNGS. mNGS and tNGS detected more MTBC than Xpert-MTB. Meanwhile, mNGS detected more non-tuberculous mycobacteria (NTM) than tNGS. Moreover, SARS-CoV-2 was genotyped, and NTM was detected and identified at the species level, which showed that mp-tNGS could identify more samples with a low pathogen burden, whereas hc-tNGS could be used to identify more precise subtypes ([Supplementary Tables S10 and S11](#)).

Discussion

In this study, we introduced the main development process and key issues of the two tNGS methods for

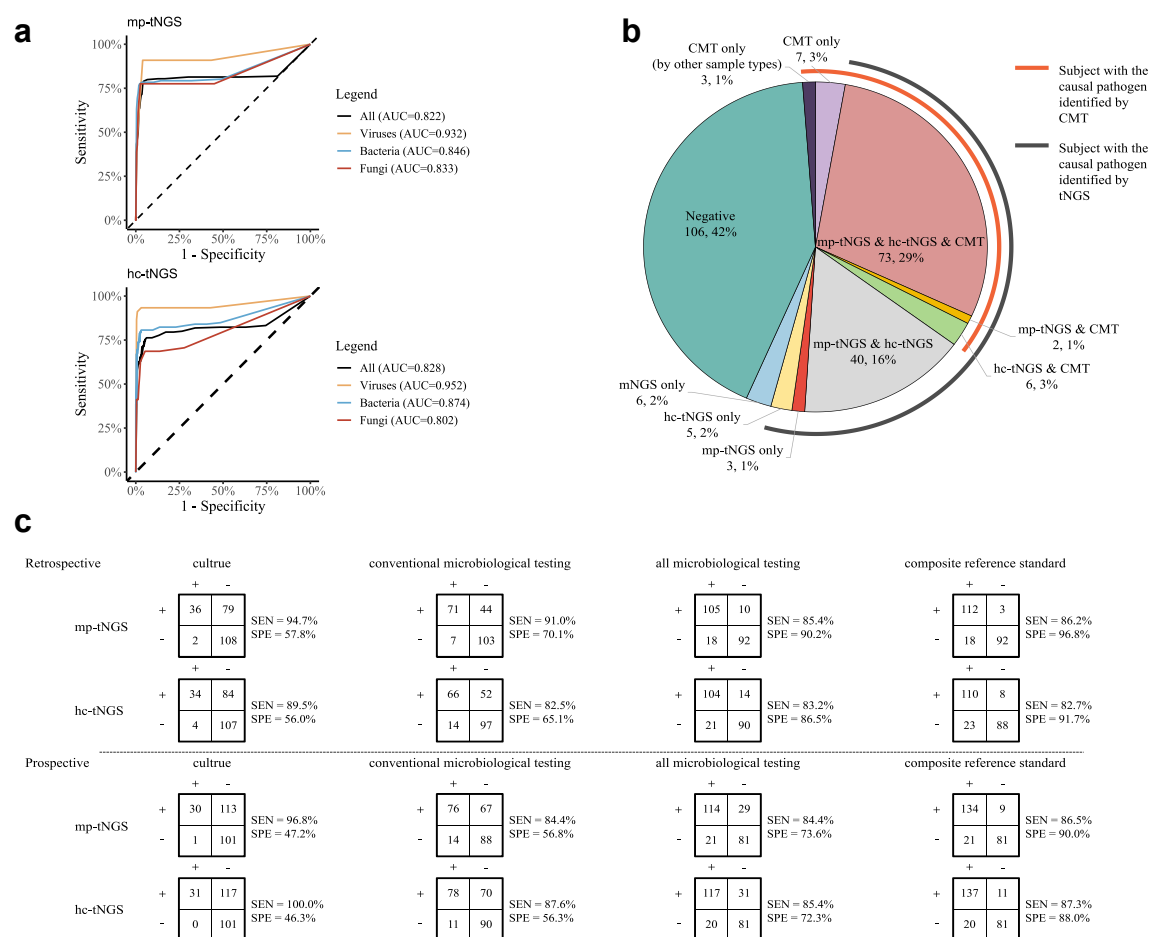


Fig. 3: Evaluation of clinical diagnostic efficacy of mp-tNGS and hc-tNGS methods. (a) Receiver operating characteristic (ROC) curves showing the diagnostic value of mp-tNGS and hc-tNGS methods in detecting pathogens in 229 retrospective samples. Statistical methods were according to the algorithm in Gu, W. et al., 2021.⁵ (b) Pie chart showing the proportion of samples identified as causal pathogens by the mp-tNGS and hc-tNGS in the prospective study (n = 251). CMT, conventional microbiological testing. (c) Contingency tables for hc-tNGS results, mp-tNGS results vs. clinical culture, conventional microbiological testing, and composite reference standard in retrospective and prospective studies (n = 480). Adjudication rules of true positive (TP), false positive (FP), true negative (TN), and false negative (FN) at the sample level were shown in [Supplementary Fig. S3b](#). A detailed list of results is available in [Supplementary Table S5](#). SEN, sensitivity; SPE, specificity.

identifying respiratory pathogens, including similarities and differences in analytical performance. Both mp-tNGS and hc-tNGS showed good analytical performance (LoD, linearity, precision and interference), which was systematically evaluated using simulated samples. For hc-tNGS, similar to mNGS, some interference was observed from the human background, and a small amount of interference from high genomic-similarity microorganisms was caused by the fault tolerance of the probe. For mp-tNGS, similar to multi-qPCR, high concentrations of pathogens produced competitive inhibition. In clinical studies, all microbiological tests and clinical adjudications were used as a composite reference standard, and the accuracy of both tNGS methodologies was fully validated through retrospective and prospective studies. The normalized reads

of different NGS methods (both tNGS and mNGS) are important for the clinical judgment of whether it is a causal pathogen.

Workflow differences among mp-tNGS, hc-tNGS, and mNGS resulted in variability in cost and diagnostic performance, which are summarized in [Table 3](#). Both mp-tNGS and hc-tNGS are much cheaper than mNGS and do not require human genome deletion steps with simultaneous DNA and RNA dual-process detection. Given its low cost and good performance on clinically significant pathogens, tNGS may be a potential pre-choice for mNGS in patients with uncritical LTRI.

In this study, mNGS failed to detect *P. jirovecii* at a higher rate than tNGS. The lower sensitivity of *P. jirovecii* detection by mNGS was attributed to trophoblasts destroyed by the host-removal step of

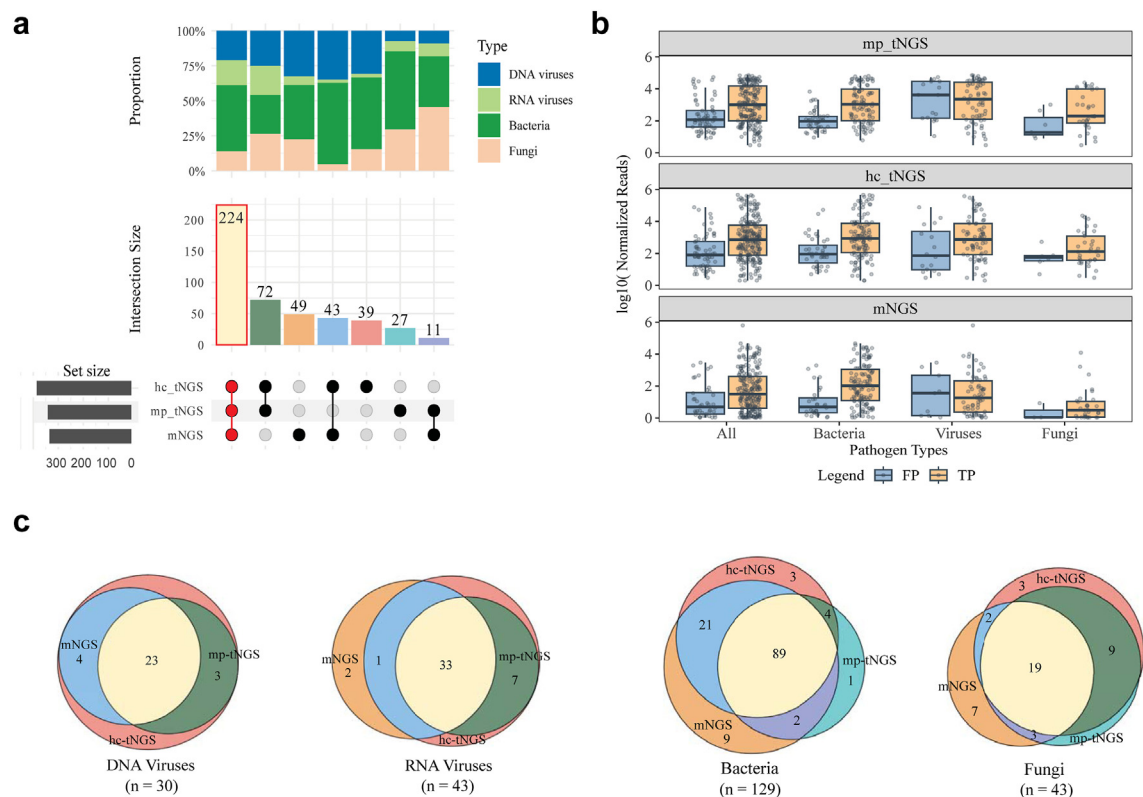


Fig. 4: Detection performance of pathogens using tNGS and mNGS methods in prospective study (n = 251). (a) Count and intersection size of pathogens detected by three NGS methods. (b) Box plot showing the normalized reads in the causal pathogen group (TP) compared with that in the non-causal pathogen group (FP). (c) Venn plot showing the overlap of causal pathogens detected among mp-tNGS, hc-tNGS, and mNGS.

differentiation cleavage. Notably, mNGS showed much higher detection rates for *P. aeruginosa*, *S. maltophilia*, and NTM than tNGS. *P. aeruginosa* and *S. maltophilia* are common reagent contaminants, which are a challenge in NGS approaches. tNGS could balance enrichment efficiency and contamination or control reagent background to address this problem in the future. For NTM, no favored unique gene probe could correctly identify NTM species, resulting in the low sensitivity of tNGS. All existing commercial tests detect a limited number of species or species complexes and have sub-optimal specificity.^{28,29} These limitations can be addressed by increasing the number of targeted probes or improving the knowledge of genomic features. Increased detection of TB by mNGS and tNGS compared to Xpert-MTB may be associated with a low burden of pathogens. In initial analytical studies, in which a LoD of 131 CFU/mL, with which Xpert-MTB detected *M. tuberculosis* in sputum,³⁰ could help to explain this.

The sensitivity was higher in the prospective study than in the retrospective one, whereas the specificity was lower. This was attributed to the samples in the prospective study being fresher and not freeze-thawed. Further analysis of the clinical feedback information and more sensitive detection in a prospective study may

help build more effective models and thresholds in the future.

According to analytical performance studies, in the context of samples with a high proportion of host nucleic acids, host depletion techniques should be contemplated for hc-tNGS before the construction of sequencing libraries. The tolerance of the probe enables hc-tNGS to enrich and detect pathogens not included in the defined target range. Meanwhile, mp-tNGS needs to set the panels according to different syndromes, avoiding the competitive inhibition caused by abundant colonized microbes in various sites. Similar to mNGS, reagent- and laboratory-associated contaminants are also a challenge for tNGS. Controlling the concentration of background bacteria is very important for NGS methods; however, tNGS can avoid false-positive pathogens by adjusting the concentrations of primers and probes, leading to an increase in LoD. Furthermore, tNGS can be made more sensitive to important pathogens of clinical concern by adjusting the concentrations of primers and probes. In the target selection of the mp-tNGS method in this study, most of anaerobic bacteria were not included, mainly because anaerobic bacteria are normal human oral colonizing bacteria. Recent



Fig. 5: Heat map showing consistency among the NGS methods, clinical judgments and conventional testing (Xpert MTB/RIF assays [Xpert-MTB], bronchoalveolar lavage fluid [BALF] galactomannan assays [GM], and cryptococcal antigen (CrAg) testing) in detecting several pathogens of clinical concern. The red, light blue, and gray color blocks indicate positive, negative, and undetected, respectively. MTBC, *Mycobacterium tuberculosis* complex; NTM, non-tuberculous mycobacteria.

studies have shown that the most common pathogens of aspiration pneumonia are *S. aureus*, *S. pneumoniae*, *Klebsiella* and *E. coli* (this part has been included in the panel), and the anaerobes have been recovered less frequently.³¹ Moreover, if too many anaerobic targets were involved in mp-tNGS panel, amplification bias would be easily caused. In conclusion, tNGS is a flexible panel that allows the establishment of a reasonable range of pathogens with different enrichment efficiencies suitable for different specimen types and syndromes.

In this study, compared with CMTs, tNGS had better performance in the detection of MTBC, NTM, *P. jirovecii*, *Mucorales*, *Nocardia*, *L. pneumophila*, etc (Supplementary Table S9). The main reason is that these pathogens are usually clinically difficult to culture or fall beyond the pathogen detection range or detection sensitivity threshold of PCR methods. Additionally, tNGS also had better detection rate than CMTs and could identify high genomic-similarity microorganisms.

Remarkably, it is worth mentioning that the timely identification of tuberculosis with other pathogens by tNGS method has very important clinical and epidemiological significance for early detection and triage of patients with tuberculosis in general hospitals. Furthermore, because of DNA and RNA co-detection workflow of tNGS, tNGS offers better diagnostic value in patients suspected of RNA virus infections or suspected of co-infections involving both RNA and DNA pathogens. Nevertheless, it is known that mNGS can identify rare pathogens, which are difficult by other methods. Therefore, mNGS remains an excellent recommendation for patients suspected of rare pathogen infections or those presenting with severe disease conditions.

Numerous studies and reviews have raised concerns regarding tNGS. Firstly, interpreting pathogen reports derived from tNGS is still challenging. The tNGS panel employed in this study covers a broad spectrum, and large tNGS panels can even assess thousands of genes

Type	Event	mp-tNGS	hc-tNGS	mNGS
Base features	Target spectrum	198 species	>3000 species	>20,000 species
	Pathogen coverage (BALF)	Nearly all covered	Almost all covered	All covered
	Simultaneous detection of DNA and RNA	Yes	Yes	No, required individual procedures
	Enrichment	Multiplex PCR	Hybridization probe capture	Host depletion
	Sequencing data size	100 K (0.1 M)	1 M	20 M
	Turnaround time	10.3 h	16–23 h	16–24 h
Performance metrics	Interference	Target detection may be interfered by high titers of other pathogens	Target detection interfered by high host genetic background and high genomic-similarity microorganisms	Target detection interfered by high host genetic background and high genomic-similarity microorganisms
Comparison of detection performance on BALF samples	Main threshold parameters	Normalized reads: RPhK	Normalized reads: RPM	Normalized reads: RPM
	Reference parameters for interpretation	Coverage, confidence, baseline of controls	Relative abundance, distribution dispersion, baseline of controls	Relative abundance, distribution dispersion, baseline of controls
	Excels in detecting targets:	<i>P. jirovecii</i> , <i>A. fumigatus</i> , <i>C. neoformans</i> , MTBC, <i>Norcardia</i> spp., RNA viruses	<i>P. jirovecii</i> , <i>A. flavus</i> , <i>C. neoformans</i> , MTBC, <i>Norcardia</i> spp., EBV, CMV, RNA viruses	<i>A. fumigatus</i> , <i>A. terreus</i> , <i>C. neoformans</i> , MTBC, <i>Norcardia</i> spp., NTM, DNA viruses, and other rare pathogens
	Weak at detecting targets:	<i>P. aeruginosa</i> , <i>S. maltophilia</i> , EBV, CMV, NTM, <i>Mucorales</i> , anaerobic bacteria	<i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>A. fumigatus</i> , NTM, <i>Mucorales</i>	<i>P. jirovecii</i> , <i>S. pneumoniae</i>
	Interpretation for identifying the pathogens	Relatively easy	Require proficiency in microbiology and interpretation	Require proficiency in microbiology and interpretation
Others	Cost	~120 USD	~300 USD	DNA procedure: ~500 USD DNA + RNA procedure: ~900 USD
	Inadequacy	Long research and development cycle, limited targets, need to accumulate enough samples for sequencing	Complex experimental procedures, longer turnaround time	High cost, require individual procedures for DNA and RNA detection, longer turnaround time

Abbreviation: RPhK, The number of reads per 100,000 sequencing reads; RPM, The number of reads per one million sequencing reads; MTBC, *Mycobacterium tuberculosis* complex; NTM, Non-tuberculous mycobacteria; CMV, Human herpesvirus 5 (cytomegalovirus); EBV, Human herpesvirus 4 (Epstein-Barr virus); USD, USA dollar.

Table 3: Technical and practical comparison among NGS methods.

in parallel,³² which typically leads to a preliminary report with multiple microbes, encompassing pathogenic and opportunistic pathogens, and colonizing microorganisms. The distinction between colonizing or background microbes and infectious pathogens often lacks clarity. Discriminating clinically relevant details from this extensive list demands meticulous assessment. Doctors need to synthesize other microbiological tests and clinical information to make a comprehensive judgment and ultimately reach a conclusion. Secondly, although the PCR procedure before sequencing increases the detection sensitivity of the low-abundant microbes, it inversely increases contamination risk, let alone the potential risk of contaminant introduction during the steps in subsequent NGS procedures.³³ Such contamination would confound the results, making interpretation difficult. Nonetheless, this issue can be mitigated by incorporating non-template or negative control samples for quality monitoring and control purposes, which was incorporated in our tNGS protocol. The establishment of a tNGS panel requires thorough threshold investigation and sophisticated analytical performance evaluation, which can significantly enhance our understanding

of the advantages and limitations of tNGS. Therefore, it is essential to employ prospective research to strengthen the communication between physicians and laboratory technicians regarding report interpretation.

In 2023, the WHO officially enlisted tNGS in the list of recommendation methods for tuberculosis and drug resistance bacteria identification,^{34,35} and discussed its potential use in low-income countries. We consider this to be a significant signal that tNGS received acceptance in infection diagnosis, and tNGS may continue to receive increasing recognition and application in the future. Previous studies used tNGS for pathogen detection in sepsis,³⁶ pneumonia,^{37–39} and periprosthetic joint infection.¹¹ However, most of the reported tests applied a PCR amplification method, targeting the 16S, 23S, and 28S rRNA genes.^{11,36,39} A report targeted 153 pathogens assays using mp-tNGS, which was as effective as mNGS in detecting respiratory pathogens but lacked *Mucorales* without RNA workflow or clinical adjustment.³⁷ The hc-tNGS panel performed for BALF in a previous study showed a higher LoD (10^2 – 10^4 CFU/mL)³⁸ than the LoD (50–450 CFU/mL) in our study, with a much narrower pathogen spectrum and lower detection of *P. jirovecii*.

This study had some limitations. First, the number of organisms tested was limited, resulting in an inadequate evaluation of certain types of pathogens. Secondly, orthogonal testing, such as qPCR, antigen assays, and Xpert-MTB, was not performed on all samples in parallel. Third, the effects of mp-tNGS and hc-tNGS on patient prognoses were not evaluated.

In conclusion, we systematically evaluated the efficacies of mp-tNGS and hc-tNGS for pathogen detection in BALF samples. The pathogen spectrum of tNGS could cover more than 95% of the cases. These results suggest that tNGS has large clinical application prospects for diagnosing LTRI in most situations owing to its lower cost and good detection ability. These data may also contribute to a more comprehensive understanding of tNGS, and provide reference and guidance for the development, technical reference and clinical application of tNGS assays.

Contributors

Y.Y., P.Z., C.H., and H.W. conceived the study. Y.Y., Y.G., H.C., S.M., and L.S. collected the clinical data for the study. Y.Y., P.Z., Y.L., J.L., C.H., and H.W. established and optimized the experimental protocols. Y.Y. and P.Z. verified the underlying data and performed data analysis. Y.Y., P.Z., and H.W. prepared the first draft of the manuscript. All authors were responsible for data interpretation, critical revision of the manuscript, and approval of the version submitted for publication.

Data sharing statement

The fastq files containing the microbial sequences from the experiments can be found in the SRA database (<https://www.ncbi.nlm.nih.gov/sra>) under accession number SUB14206958.

Declaration of interests

P.Z., Y.L., J.L., and C.H. are employees of Guangzhou KingCreate Biotechnology Co., Ltd. The other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2024.105307>.

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