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Original Article

Efficacy of targeted next generation sequencing for pathogen detection in lower respiratory tract infections

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Abstract: Objective: To investigate the diagnostic utility of targeted next-generation sequencing (tNGS) in the diagnosis of lower respiratory tract infections. Methods: Patients with lower respiratory tract infection in East Area of Yantai Yantaishan Hospital from December 2021 to September 2023 were retrospectively analyzed. Sputum samples were tested using both tNGS technology and conventional microbiological examination. Data were collected on general clinical features and test outcomes. The study evaluated the efficacy of tNGS by comparing its positive detection rate against traditional methods and analyzing detection differences among patients with varying clinical characteristics. Receiver operating characteristic (ROC) analysis was used to determine the diagnostic accuracy of both testing methods. Results: A total of 281 patients were included, with corresponding sputum specimens. The tNGS method showed a higher positivity rate of 90.0%, significantly outperforming the conventional method's rate of 70.82% ($P<0.05$). Among 199 patients with concordant positive results, 38.22% fully agreed, while 53.40% completely disagreed between the two methods. Mycobacterium tuberculosis, Candida albicans, and Pseudomonas aeruginosa were the most frequently detected pathogens, respectively. tNGS significantly reduced the time required for pathogen detection ($P<0.001$) and identified a higher rate of mixed infections compared to conventional methods (49.11% vs 2.85%, $P<0.001$). Positive tNGS detection rates significantly differed between patients with abnormal vs normal C-reactive protein or procalcitonin levels. The AUC for tNGS was 0.867, indicating superior diagnostic accuracy over the conventional method ($P<0.05$). Conclusions: tNGS technology demonstrates a high positivity rate and rapid pathogen detection in lower respiratory tract infections, with notable advantages in identifying mixed infections. This method shows potential for enhancing diagnostic accuracy and treatment decisions in clinical settings.

Keywords: Lower respiratory tract infection, targeted next generation sequencing, conventional microbiological test, pathogenic diagnosis

Introduction

Infectious diseases account for approximately 25% of all global mortalities annually, totaling over 17 million deaths [1]. Among these, lower respiratory tract infections are a leading cause of mortality [2]. Accurate pathogen detection is crucial for precise diagnosis and effective treatment strategy formulation for these infections [3, 4]. Without clear identification, targeted drug treatment becomes challenging, potentially delaying disease improvement and exacerbating symptoms, sometimes fatally.

Traditionally, pathogen identification in lower respiratory tract infections has relied on methods such as microbial culture, polymerase chain reaction (PCR), and antigen or antibody immunological tests. However, microbial culture is time-consuming and has a low detection rate, particularly for anaerobes, fastidious bacteria, and viruses which are challenging to culture [5]. Although antigen detection is simple and specific, its sensitivity is low and a negative result does not necessarily rule out an infection [6]. Antibody tests are influenced by the infection duration, often resulting in false negatives, especially in immunocompromised patients [7]. Additionally, both antigen/antibody immunology and PCR are limited to detecting pathogens with known genetic sequences, leaving those with unknown sequences undetected [8].

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In recent years, next-generation sequencing (NGS) has emerged as a rapid, high-throughput method. Targeted next-generation sequencing (tNGS), in particular, offers higher sensitivity and cost-effectiveness than metagenomic sequencing. It addresses both DNA and RNA, reduces interference from host nucleic acids, and is especially effective in detecting low-concentration pathogens. A retrospective study involving 35 children with traumatic brain injuries or suspected post-surgical intracranial infections showed that tNGS confirmed infections in 18 cases, demonstrating its high diagnostic accuracy for central nervous system infections in pediatric neurosurgical patients [9]. Clinical literature on the application of tNGS in lower respiratory tract infections remains scarce, predominantly consisting of case reports or small-scale retrospective studies. This study aims to evaluate the accuracy and sensitivity of tNGS by comparing it with traditional methods, with the findings anticipated to underscore tNGS's high sensitivity and accuracy, thereby establishing its diagnostic value for lower respiratory tract infections.

Material and methods

Subjects

This retrospective analysis included patients with lower respiratory tract infections admitted to the Department of Pulmonary and Critical Care Medicine at Yantai Yantaishan Hospital from December 2021 to September 2022. All patients underwent both tNGS and conventional microbiological tests. A comprehensive review of the clinical data for each patient was performed. The study received approval from the Ethics Committee of Yantai Yantaishan Hospital (Approval number: No. 2019-033).

Inclusion Criteria: (1) Patients diagnosed with lower respiratory tract infections, as defined by the European guidelines for the diagnosis and treatment of lower respiratory tract infections in adults [10], supplemented with clinical data, and symptoms such as cough, expectoration, fever, and dyspnea, signs like pulmonary rales, and evidence from blood infection indicators and chest radiographs. (2) Age range between 18 and 75 years. (3) Both tNGS and conventional microbiological tests for pathogenic diagnosis were conducted. (4) Complete clinical data were available.

Exclusion Criteria: (1) Patients who declined sample collection. (2) Sputum samples that did not meet the quality standards required for tNGS. (3) Patients with incomplete clinical data.

Sample collection

Adequate sputum specimens were collected following proper protocols. Oral hygiene was performed in the morning, after which patients were instructed to take a deep breath and forcefully expel sputum from the respiratory tract. The sputum specimens were stored in sterile containers. For patients unable to produce sputum through coughing, sputum was collected using a disposable suction tube under negative pressure. Alternative methods, such as sputum induction or tracheal aspiration, were also employed. Approximately 3 mL of sputum was collected and stored at -20°C within 48 hours for tNGS analysis. Residual sputum was utilized for conventional microbiological tests.

Conventional microbiological tests

As described in previous studies [11], sputum specimens were divided into aliquots for comprehensive pathogen detection. One aliquot was used for bacterial and fungal smear and culture. Another aliquot was dedicated to the Fungal-D glucan test and the Aspergillus galactosidase test. A further aliquot was allocated for antibody testing against nine respiratory pathogens, including Legionella pneumophila, Mycoplasma pneumoniae, Chlamydia pneumoniae, respiratory syncytial virus, adenovirus, Rickettsia, influenza virus, parainfluenza virus, and cytomegalovirus using real-time PCR. Additionally, DNA sequences specific to various pathogens such as Streptococcus pneumoniae, Staphylococcus aureus, methicillin-resistant Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Stenotrophomonas maltophilia, Haemophilus influenzae, and Mycobacterium tuberculosis were detected.

Targeted next-generation sequencing

For tNGS, one aliquot of sputum sample was mixed with sputum digestive buffer in a 1.5 mL tube and centrifuged. The mixture was then homogenized for 30 seconds using a vortex mixer. DNA was extracted from 500 μL of the

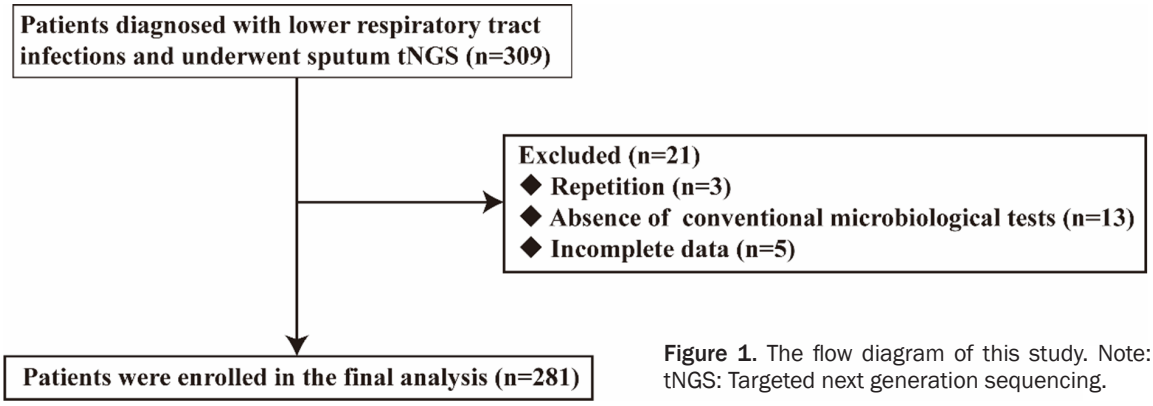

Figure 1. The flow diagram of this study. Note: tNGS: Targeted next generation sequencing.

homogenate using DNA Miniprep Kits (Monarch International LTD., USA, Lot number: T1010S) following the manufacturer's protocol. The DNA library for targeted sequencing was prepared by PCR amplification using Respiratory Pathogen Detection Kits (Shanghai Boke Biotechnology Co., Ltd., China, Lot number: BK-P63368).

cDNA was synthesized via reverse transcription under the following conditions: 25°C for 5 minutes, 37°C for 45 minutes, and 85°C for 5 seconds, then held at 4°C. The cDNA products were enriched targeting specific regions with a PCR cycle set at 95°C for 3 minutes, followed by 28 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 1 minute and then held at 4°C. PCR products were centrifuged briefly, and magnetic beads were used to purify the targeted PCR products.

Library amplification was conducted on the purified PCR products, followed by another brief centrifugation. The library was then cleaned using magnetic beads and quantified using a Qubit 4.0 fluorometer. The prepared library was diluted, denatured, and sequenced using Illumina MiSeq Reagent Nano Kits (Illumina, Inc., USA, Lot number: MS-103-1001) on the KM MiniseqDx-CN platform.

Data management and analysis were performed using a system that initially identified raw data sequences by their adapters. Quality control was enforced by retaining reads where the double-ended length exceeded 60 bp and with a quality score (Q30) ≥75%. Primers were identified, and reads correctly aligned at both ends were retained. Final reads were compared against pathogen databases, including the GenBank, Nucleotide, and RefSeq databases from NCBI, for pathogen identification.

Statistical analysis

Data analysis was performed using SPSS software (IBM, USA), version 23.0. Measurement data were presented as mean ± standard deviation (SD). Comparisons between the two groups were conducted using independent samples t-tests. Enumeration data were expressed as cases/percentage [n (%)], and comparisons between groups were made via Chi-square tests or McNemar tests as appropriate. Receiver operating characteristic (ROC) analysis was conducted, and the area under the ROC curve (AUC) was calculated to evaluate the diagnostic accuracy of tNGS compared to conventional microbiological methods. A *P*-value <0.05 was considered indicative of statistically significant differences.

Results

Characteristics of patients

Initially, 309 patients diagnosed with lower respiratory tract infections and undergoing tNGS were considered for enrollment in this study. Of these, 21 were excluded due to the absence of conventional microbiological tests, duplications, and incomplete data. Consequently, a cohort of 281 patients meeting the inclusion criteria was analyzed (**Figure 1**).

Table 1 shows that the average age of the patients was 65.7±8.5 years old, with 176 (62.63%) being male. Comorbidities such as diabetes mellitus, hypertension, and heart dis-

Table 1. Clinical characteristics of patients included in this study

Parameters		Values
Gender (cases)	Male	176
	Female	105
Age (years)		65.7±8.5
Expectoration (cases)		220
Cough		194
Fever		98
Diabetes mellitus		29
Hypertension		42
Heart disease		20
White blood cell count ($\times 10^9$/L)		7.39
CRP (mg/L)		30.52
PCT (ng/mL)		0.28

Note: CRP: C-reactive protein. PCT: Procalcitonin.

ease were present in 10.32%, 14.95%, and 7.12% of patients, respectively. Clinical symptoms confirmed included cough (69.04%), expectoration (78.29%), and fever (34.88%). Hematological indices revealed an average white blood cell count of 7.39×10^9/L, C-reactive protein (CRP) at 30.52 mg/L, and procalcitonin (PCT) at 0.28 ng/mL.

Pathogen detection results of tNGS

As illustrated in **Figure 2**, among the 281 patients analyzed, tNGS achieved an overall microbial detection rate of 90% (253/281). The most commonly identified fungi were Candida albicans (27 cases) and Aspergillus (24 cases). The most prevalent bacteria included Mycobacterium tuberculosis (30 cases), Pseudomonas aeruginosa (26 cases), and Klebsiella pneumoniae (24 cases). Other notable pathogens were Pneumocystis jejuni (22 cases), Haemophilus influenzae (21 cases), Acinetobacter baumannii (18 cases), Staphylococcus aureus (16 cases), nontuberculous mycobacteria (12 cases), Stenotrophomonas maltophilia (12 cases), and Streptococcus pneumoniae (11 cases). The top viruses detected were human herpesvirus type 4 (31 cases), human herpesvirus type 7 (17 cases), and rhinovirus (15 cases).

The detection rates of bacteria, fungi, viruses, mycoplasma, and Mycobacterium tuberculosis by tNGS were 60.5%, 24.2%, 28.47%, 12.46%, and 10.68%, respectively. These rates were significantly higher than those achieved by conventional microbiological methods, with statistical differences observed between the two groups for all pathogen types (all $P<0.05$, **Figure 3**).

Comparison of positive results between tNGS and conventional microbiological tests

As shown in **Table 2**, among the 281 patients with lower respiratory tract infections, 191 showed positive results for both tNGS and conventional microbiological tests, while 20 cases were negative in both. tNGS alone identified 62 positive cases, and conventional tests alone identified 8. Of the 191 double-positive cases, 73 showed complete consistency between tNGS and conventional tests, 16 showed complete inconsistency, and 102 showed partial consistency (**Figure 4**). The positive detection rate for conventional microbiological tests was 70.82% (199/281), significantly lower than that for tNGS. The Kappa value was 0.259, indicating poor consistency between the two testing methods.

Comparison of mixed infection identification and test time between tNGS and conventional tests

tNGS detected mixed infections in 49.11% (138/281) of cases, significantly higher than the 2.85% (8/281) detected by conventional microbiological tests, with statistically significant differences ($P<0.001$). Moreover, tNGS required an average of 48.73±2.64 hours per test, compared to 71.25±19.85 hours for conventional tests, highlighting significant time-efficiency with tNGS (**Figure 5**).

Comparison of positive tNGS detection across different patient characteristics

Table 3 presents that significant differences were observed in clinical characteristics such as CRP and PCT levels when comparing positive and negative tNGS results (all $P<0.05$). No statistical differences were found for other characteristics.

Comparison of diagnostic values of tNGS and conventional microbiological methods

The diagnostic performances of tNGS and conventional microbiological methods were evaluated using ROC analysis. The AUC for conventional methods was 0.776 (95% CI: 0.710-0.812), while for tNGS it was 0.867 (95% CI:

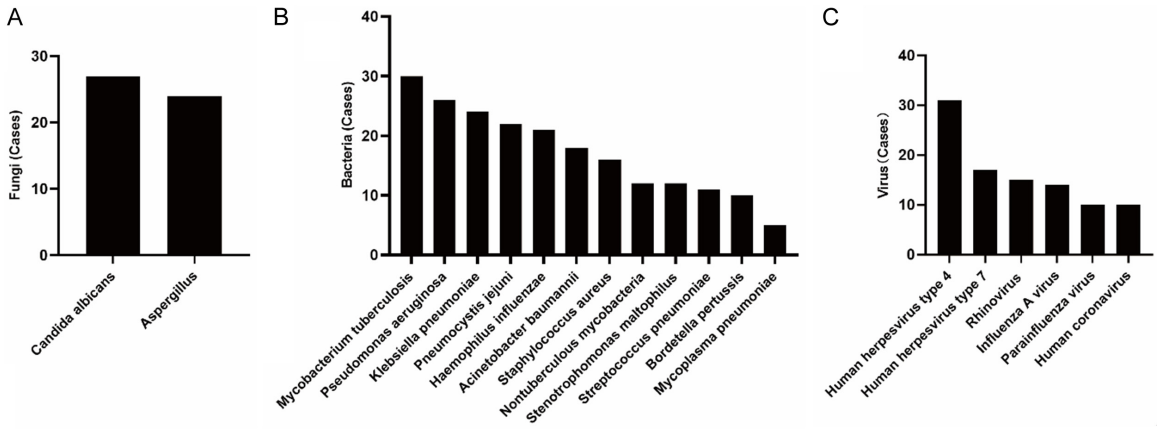

Figure 2. Distribution of potential pathogens in this study. A: The distribution of fungi. B: The distribution of bacteria. C: The distribution of virus.

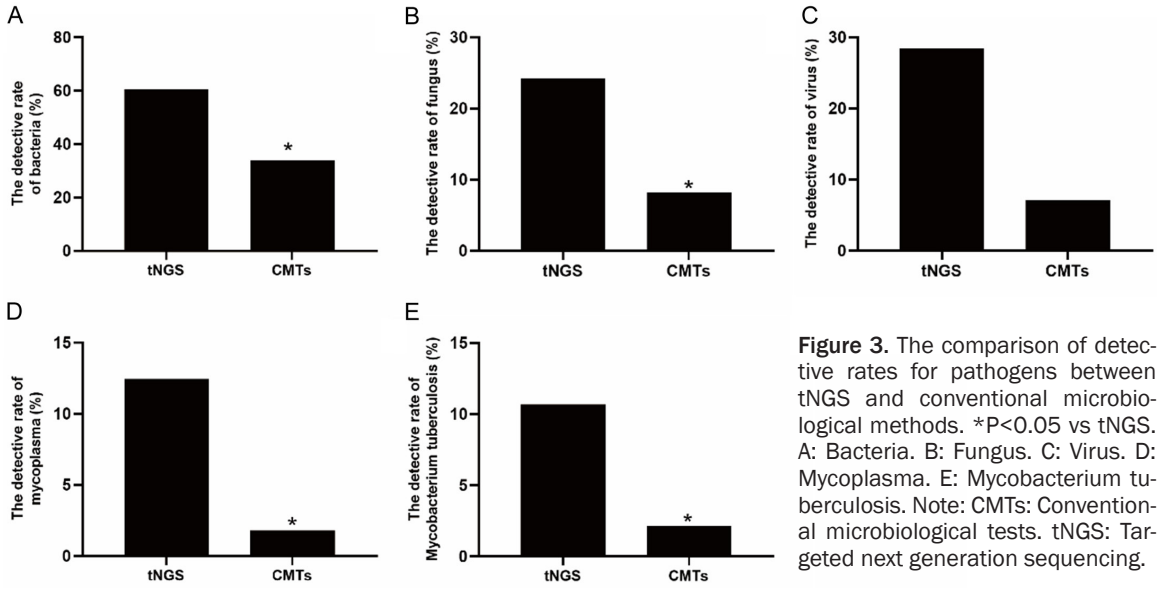

Figure 3. The comparison of detective rates for pathogens between tNGS and conventional microbiological methods. *P<0.05 vs tNGS. A: Bacteria. B: Fungus. C: Virus. D: Mycoplasma. E: Mycobacterium tuberculosis. Note: CMTs: Conventional microbiological tests. tNGS: Targeted next generation sequencing.

Table 2. The comparison results of tNGS and conventional microbiological tests

tNGS tests (cases)	Conventional microbiological tests (cases)		Total (cases)
	Positive	Negative	
Positive	191	62	253
Negative	8	20	28
Total (cases)	199	82	281

Note: tNGS: Targeted next generation sequencing.

0.795-0.891), indicating superior diagnostic accuracy with tNGS. There were significant differences between the AUC values, confirming the enhanced diagnostic capability of tNGS (P<0.05, **Figure 6**).

Discussion

The tNGS method has emerged as a pivotal alternative to conventional microbiological tests for detecting microorganisms and clinically diagnosing respiratory tract infections, significantly advancing the field of infectious disease diagnostics. Previous studies have demonstrated that tNGS offers higher sensitivity compared to traditional methods for detecting bacteria in pulmonary infections [12]. tNGS not only utilizes multiplex PCR to amplify targeted genes of anticipated

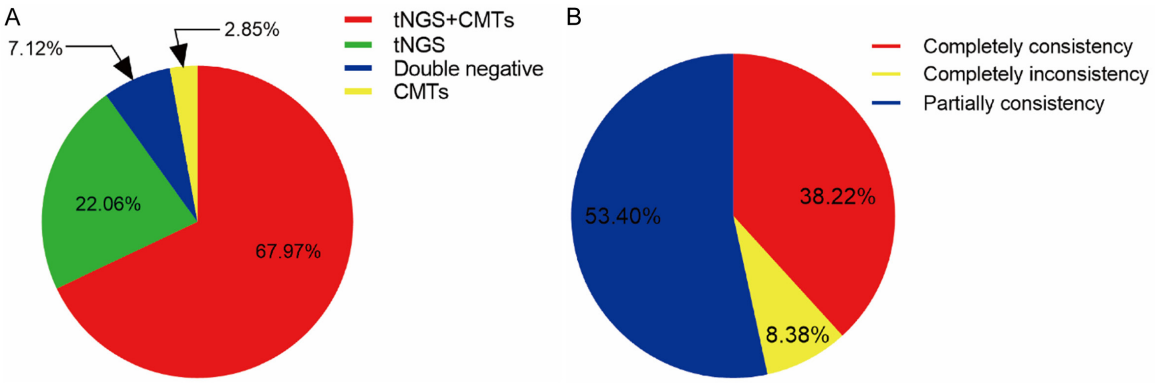

Figure 4. Consistency of pathogen detection results between tNGS and CMTs. A: The positive detection results. B: The detective results of consistency. Note: CMTs: Conventional microbiological tests. tNGS: Targeted next generation sequencing.

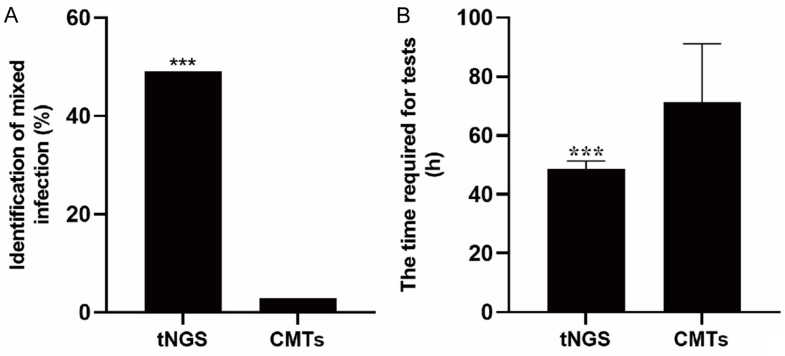

Figure 5. Comparison of identification of mixed infection and the test time between tNGS and conventional tests. A: The detective rate of mixed infection. B: The test time. ***P<0.001 vs CMTs. Note: CMTs: Conventional microbiological tests. tNGS: Targeted next generation sequencing.

pathogens but also employs second-generation sequencing technology to provide high-throughput information [13]. In clinical practice, tNGS can serve as a supplementary diagnostic tool alongside conventional microbiological tests. Unlike metagenomic sequencing, tNGS offers higher sensitivity at a lower cost [14]. However, it has been noted that tNGS relies on the prediction of microorganisms and cannot identify unknown pathogens [15]. Other studies have underscored the potential utility of tNGS in pathogen diagnosis [16].

In addition to its application in cancer diagnostics [17], this study explores the clinical use of tNGS for diagnosing lower respiratory tract infections. We employed a tNGS assay targeting 281 pathogens to assess its detection performance in sputum samples. tNGS identified a broader range of potential pathogens, including clinically relevant or common respiratory viruses, bacteria, fungi, and atypical pathogens. Compared to conventional microbiological methods, tNGS significantly enhanced the pathogen detection rate for lower respiratory tract infections, showing a higher positive detection rate (90.0% vs 70.82%). These findings indicate that many patients may harbor pathogens that remain undetected by conventional methods, echoing the results of Dai et al.'s study [18]. This comparison underscores the enhanced diagnostic capabilities of tNGS, highlighting its value in improving pathogen detection in clinical settings. Moreover, among the 191 patients who tested positive for both tNGS and conventional microbiological tests, 91.62% showed full or partial consistency between the two methods. This result highlights the promising potential of tNGS, aligning with findings from Lin et al.'s report [19]. Regarding mixed infections, it is recognized that lower respiratory tract infections often involve a complex and diverse range of pathogens and are prone to mixed infections, which conventional methods struggle to detect in a single sample. However, tNGS plays a critical role in identifying these mixed infections. Studies have shown that genome coverage rates by targeted pathogen sequence detection in mixed specimens are not compromised, with coverage rates of two

Table 3. Comparison of the positive detection for tNGS between patients with different characteristics

Parameters		Positive tNGS (Cases)	Negative tNGS (Cases)	t/χ^2	P
Gender (cases)	Male	129	47	1.398	0.237
	Female	70	35		
Age (years)	≥60	118	52	0.412	0.521
	<60	81	30		
Expectoration (cases)	Yes	150	70	3.409	0.065
	No	49	12		
Cough	Yes	140	54	0.550	0.458
	No	59	28		
Fever	Yes	70	28	0.027	0.869
	No	129	54		
Diabetes mellitus	Yes	20	9	0.054	0.817
	No	179	73		
Hypertension	Yes	30	12	0.008	0.925
	No	169	70		
Heart disease	Yes	14	6	0.007	0.933
	No	185	76		
White blood cell counts	Normal	7	3	0.003	0.954
	Abnormal	192	79		
CRP (mg/L)	Normal	3	5	4.423	0.036
	Elevation	196	77		
PCT (ng/mL)	Normal	2	7	10.630	0.001
		197	75		

Note: tNGS: Targeted next generation sequencing. CRP: C-reactive protein. PCT: Procalcitonin.

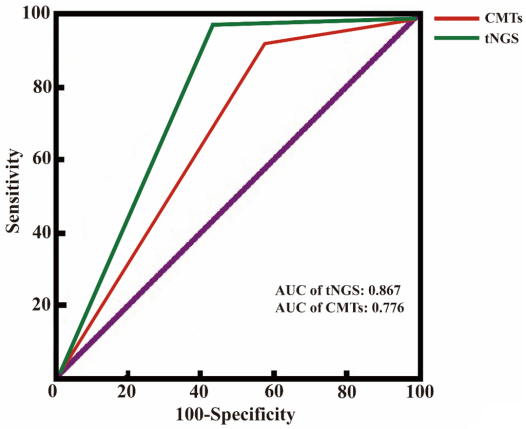

Figure 6. ROC curves for the diagnostic methods of tNGS and CMTs. Note: ROC: Receiver operating characteristic. AUC: Area under the receiver operating characteristic curve. CMTs: Conventional microbiological methods. tNGS: Targeted next generation sequencing.

viruses in two samples reaching 95%-100% [20]. This study demonstrated that the detection rate of mixed infections by tNGS was 49.11%, significantly higher than that of conventional microbiological tests, corroborating Chung et al.'s findings that tNGS offers substantial advantages in identifying mixed infections [21].

Evidence increasingly suggests that the respiratory tract is not a sterile environment and is ubiquitously inhabited by potentially pathogenic microorganisms [22]. Sputum samples collected from the lower respiratory tract via non-invasive or minimally invasive methods are likely to be contaminated by the endogenous upper respiratory tract flora [23]. Previous studies have indicated that some microbes considered pathogenic are actually part of the normal respiratory microflora [24], complicating the interpretation of tNGS results from sputum samples. Additionally, it has been reported that diagnosing an infection based solely on the detection of a specific microorganism in sputum samples is not always practical [25]. The distinction between pathogenic infection and commensal colonization typically relies on tNGS results, the clinical context of the patient, and the physician's expertise. Against this

backdrop, tNGS offers deeper insights into pathogen information compared to conventional methods. By enabling the simultaneous detection of multiple pathogens, tNGS significantly enhances diagnostic efficiency and aids physicians in improving differential diagnoses and identifying mixed infections.

This study has several limitations: First, the relatively small sample size may have impacted the performance and accuracy of tNGS. Second, due to the absence of standard reference results for pathogenic diagnosis, we were unable to calculate the sensitivity and specificity of tNGS, thus limiting a comprehensive assessment of its diagnostic performance. Third, distinguishing between microbial infection and colonization was challenging, as tNGS lacks uniform criteria for pathogen diagnosis. Fourth, the positive results from tNGS within this study were based solely on the detection of pathogen reads.

In conclusion, this study underscores the clinical utility of tNGS based on sputum samples in the diagnosis of lower respiratory tract infections. The findings demonstrate that tNGS offers a significantly higher positive detection rate and timeliness compared to conventional microbiological tests, aiding in the early identification of potential pathogens, particularly those that are rarely colonized or highly pathogenic. Additionally, tNGS provides advantages in identifying mixed infections. These results highlight the potential of tNGS for improving the diagnosis and pathogen detection in lower respiratory tract infections.

Disclosure of conflict of interest

None.

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