Article

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Comparison of Third-Generation Sequencing Technology and Traditional Microbiological Detection in Pathogen Diagnosis of Lower Respiratory Tract Infection

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Background: It is common to obtain a low detection rate and unsatisfactory detection results in complex infection or rare pathogen detection. This retrospective study aimed to illustrate the application value and prospect of the third-generation sequencing technology in lower respiratory tract infection disease.

Methods: This study recruited 70 patients with lower respiratory tract infection (LRTI). Pathogen detection of bronchoalveolar lavage fluid (BALF) from all patients was performed using nanopore metagenomic sequencing technology and traditional culture. BALF culture combined with quantitiative PCR (qPCR) was used as a reference standard to analyze the sensitivity and specificity of nanopore sequencing technology. The current study also collected the examination results of enrolled samples using technical methods sputum culture, tuberculosis DNA (TB-DNA), and Xpert MTB/RIF and analyzed the detection efficiency of nanopore sequencing for *Mycobacterium tuberculosis*.

Results: The positive rates of pathogens in 70 BALF samples detected by conventional culture and nanopore sequencing were 25.71% and 84.29%, respectively. Among the 59 positive BALF cases using nanopore sequencing, a total of 31 pathogens were identified, of which the proportions of bacteria, fungi, viruses, and other pathogens were 50%, 17%, 32%, and 1%, respectively. Using the results combined with culture and qPCR detection methods as the standard, the pathogen detection of BALF using nanopore sequencing had a sensitivity of 70% and a specificity of 91.7%. Additionally, the positive rate of the detection of *M. tuberculosis* using nanopore sequencing was 33.3% (6/18). The clinical medication plans of 74.3% (52/70) of the patients were referred to the nanopore sequencing results, of which 31 cases changed their treatment strategy, 21 supported the previous treatment plans, and 90% (47/52) of the patients finally had clinical improvement.

Conclusions: BALF detection using nanopore sequencing technology improves the process of detecting pathogens in patients with LRTI, especially for *M. tuberculosis*, fungi, and viruses, by reducing the report time from three days to six hours. The clinical application prospect of nanopore sequencing technology is promising in the pathogen diagnosis of LRTI.

Keywords: nanopore sequencing technology; lower respiratory tract infection; pathogen diagnosis; clinical application

Introduction

Respiratory tract infections seriously threaten human health. There is a wide range of pathogenic organisms, including bacteria, viruses, fungi, and *Mycoplasma pneumoniae*. However, clinical manifestations of the same infected pathogen are heterogeneous, posing a significant challenge for clinicians in making rational treatment decisions. Lower respiratory tract infection (LRTI) is the most common complication of hospitalized patients and one of the leading causes of mortality in China. Approximately 10 million new cases of LRTI are reported annually in China, with an incidence around 75/100,000 and a mortality of 30%. Among them, the differential diagnosis of community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP) is difficult due to the limitations of traditional methodologies, lack of definite etiological evidence, and changes in pathogens. The etiology of more than 30% of LRTI cases is unclear [1,2]. Although there are various methods for detecting traditional

pathogenic microorganisms, low detection rates, poor comprehensiveness, and long cycles are common problems in complex infections or rare pathogen detection. In terms of bacterial and fungal detection, culture is the primary traditional detection method, which takes 3–5 days until the report is available. Virus detection is mainly based on PCR and serological methods, and the targets are relatively fixed with certain preferences. The detection rate of bacteria and viruses using the traditional method is only 11%, and even after 30 days verification, it is 19% [3]. In 15–25% of respiratory infections, the etiology cannot be identified by conventional testing methods [4,5]. Although traditional serology and PCR detection methods have certain advantages in specificity, sensitivity, and detection periods, both can detect only a single specific pathogen at a time with low throughput, and the detection objects are limited to specific known pathogens, failing to identify unknown pathogens.

High-throughput sequencing technology has been developing for over a decade, the sequencing time has been greatly shortened, and the cost has been reduced beyond Moore's Law [6,7], giving rise to new ideas for the detection of pathogenic microorganisms. High-throughput sequencing technology can be directly used to perform whole genome sequencing after the extraction of nucleic acids from clinical samples. Pathogens are identified by comparing sample sequences to the pathogenic microorganism database. The technique can comprehensively detect the sample pathogenic microorganisms efficiently and objectively, without the need for specific amplification of a pathogen or microbial culture. Metagenomics technologies currently include second- and third-generation sequencing techniques, both of which can achieve high-throughput pathogen detection. As the third-generation sequencing, nanopore sequencing technology is characterized by a short detection period (as fast as six hours) and long read length (>1000 bp, up to 4 Mb), which effectively improves the efficiency and accuracy of shearing and assembling. It fills the genomic sequencing gap that is difficult to complete on the short-read length platform of second-generation sequencing. Though nanopore sequencing technology has great advantages in the detection of structural variation of drug resistance genes, its base-reading accuracy is slightly less than that of second-generation sequencing [8,9]. As third-generation sequencing technology has advanced in recent years, the accuracy of base judgment has come to approach 99.99% [10]. It is therefore considered an important direction for future sequencing development.

In the past, metagenomic testing was merely used as an ultimate means of pathogen detection clinically. However, as clinical practice experience increases and research data accumulate, both the efficiency and accuracy of pathogen detection have improved. The threshold of metagenomic detection may gradually change in the future to make it the method of choice in the diagnosis and treatment of clinical infectious diseases. As third-generation sequencing has the technical advantages of long read length, flexibility, and convenience, it can improve the identification accuracy of infectious pathogens and shorten pathogen detection time. However, due to the lack of supporting data for the clinical application of nanopore sequencing technology in infectious pathogens, the clinical application and popularization of this technology remain limited. Therefore, the current study applied traditional culture, quantitiative PCR (qPCR), tuberculosis DNA (TB-DNA), and Xpert MTB/RIF microbial detection methods to explore the real-world situation of third-generation sequencing technology identifying LRTI pathogens and guiding medication decisions for patients with pulmonary infection based on sequencing results, expecting to offer a reference for the future clinical application of the technology.

Methods

Patient Recruitment and BALF (Bronchoalveolar Lavage Fluid) Sample Collection

This prospective observational study recruited 70 patients with suspected LRTI/pulmonary infection who came to the Zengcheng Branch of Nanfang Hospital of Southern Medical University between August and December 2021. The inclusion criteria included symptoms of fever, cough, expectoration, shortness of breath, dyspnea, and imaging abnormalities. Demographic and baseline characteristics, clinical manifestations, laboratory test results, and medication before sampling were investigated for making the clinical diagnosis. The diagnosis of LRTI was based on comprehensive reference standards, namely all microbiological tests and clinical judgment, and referred to the diagnostic criteria of CAP and HAP [11,12]. Patients were assigned by evaluating Pneumonia Severity Index (PSI) before treatment.

BALF Collection

First, local anesthesia was achieved by injecting 2% lidocaine 1~2 mL through the biopsy orifice into the lung segment (diseased lobe segment or right middle lobe or left upper lung lingual segment) to be lavaged in the patients. After opening the target bronchial segment or subsegment, the tip of the bronchoscope was inserted, and 37 °C sterilized normal saline was injected rapidly through the operating orifice, 20~50 mL at a time, for a total of 60~120 mL. After normal saline instillation, BALF was obtained by suction with appropriate negative pressure (below 100 mm Hg) and collected in a sterile container. The total sampling retrieval rate of BALF was more than 30%, with a total volume of BALF typically ranging from around 20~45 mL. To identify infectious pathogens in BALF specimens from the 70 patients, several methods were used, including nanopore third-generation metagenomic sequencing, traditional microbiological testing, qPCR, and a combination of microbial culture with qPCR (workflow presented in Fig. 1). The

combination of microbial culture with qPCR was used as a reference standard to analyze the sensitivity and specificity of nanopore sequencing technology.

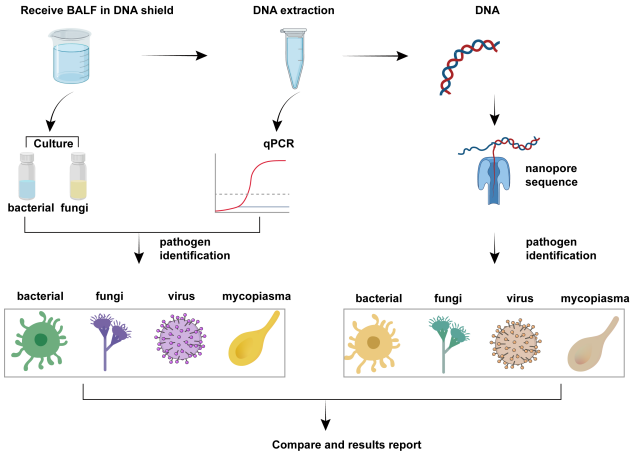

Fig. 1. Workflow of pathogen diagnosis of BALF samples from LRTI patients.

Culture and Biochemical Identification

BALF samples (100 μL) were inoculated in blood agar, MacConkey agar, and chocolate agar plates, respectively, using inoculation loops and incubated at a constant temperature of 35 °C in a 7% CO_2 environment for 24 h. Following purification and culture, the suspected pathogenic bacteria were identified using the standard biochemical method. BALF samples were also submitted for qPCR. Third-generation sequencing was applied to the remaining BALF samples after inoculation. Standard biochemical identification methods were roughly classified based on colony morphology, Gram staining, and oxidase and identified subsequently using a fully automatic microbial identification system (Vitek 2 Compact, BioMerieux, Lyon, France).

Sample Preparation and Sequencing

The sample was mixed evenly by vibration, 1 mL of the sample was aspirated to a 1.5 mL sterile centrifuge tube and centrifuged at 12,000 rpm for 2 min. The supernatant was removed. Normal saline (IN9000, Solarbio Life sciences, Beijing, China) and selective medium (5% Tween 20 (P1379, Sigma-Aldrich, St. Louis, MO, USA) and 0.5% sodium dodecyl sulfate (L3771, Sigma-Aldrich, St. Louis, MO, USA)) were supplemented, 600 μL each, mixed well by vibration, suspended, let stand at room temperature for 30 min, and centrifuged at 12,000 rpm for 2 min. The supernatant was discarded. Following the addition of 50 μL of digestion solution (1 mol/L NaOH, pH = 7.0, S5881, Sigma-Aldrich, St. Louis, MO, USA) to each sample, precipitate was mixed evenly by pipetting, suspended, digested at 37 °C for 10 min, heated at 80 °C for 2 min, supplemented with 100 mg glass beads, and vortex vibrated at 2000 rpm for 2 min. Subsequent DNA extraction was applied (TIANamp Bacteria DNA Kit [DP302-02, TIANGEN, Beijing, China]), and operation procedures were done according to manufacturer's instructions. DNA concentration was calibrated using the Qubit reagent (Q33230, ThermoFisher Scientific, Invitrogen, Carlsbad, CA, USA). Fragmentation Mix (FRM) reaction solution (SQK-RPB004 Rapid PCR Barcoding Kit (Nanopore), Oxford Nanopore Technologies, Oxford, UK) was prepared in a 0.2 mL PCR tube, stirred mildly, and mixed evenly, at 30 °C for 5 min, 80 °C for 1 min, and cooled quickly on an ice box. After the PCR product was transferred to a 1.5 mL centrifuge tube, an equal amount of 50 μL purified magnetic beads were supplied, mixed well, let stand for 5 min at room temperature, and placed on a magnetic stand after instantaneous centrifugation. When the solution turned clear, the supernatant was discarded. Following two cycles of washing with 180 μL of 80% ethanol, the sample was instantaneously centrifuged with 3000 rpm for 1 min, placed on a magnetic stand to absorb the residual ethanol, dried at room temperature for 30 s with the lid uncovered, supplied with 10 μL of 10 mM Tris-HCl (pH = 8.0, PHG0002, SAFC Biosciences, Lenexa, KS, USA) & 50 mM NaCl (S8210, Solarbio Life sciences, Beijing, China) mixed solution, and gently rotated the suspended magnetic beads. Following incubation for two minutes at room temperature, the sample was placed on the magnetic stand. When the solution turned clear, the eluate was aspirated for later use. Qubit reagent was used to calibrate the concentration of each sample eluate. As per relevant concentrations, the same absolute quantity template was employed and mixed into 10 μL with a total concentration ranging from 100 to 200 ng/μL, supplemented with 1 μL Rapid Adapter (RAP) solution (SQK-RPB004 Rapid PCR Barcoding Kit (Nanopore), Oxford Nanopore Technologies, Oxford, UK), mixed gently, and incubated at room temperature for 15 min. Sequencing was performed when the loading mixture was ready within 15 min. The sequencing data volume was 500 Mb.

Nanopore Sequencing and Data Analysis

Raw data files were generated by the MinION sequencer (Oxford Nanopore Technologies, Oxford, UK) in fast5 format, and the real-time identification and the fastq files were completed using MinKnow software (version 1.11.5, Oxford Nanopore Technologies, Oxford, UK). Low-quality sequences were filtered using MinKnow software. Filtered data were removed from host DNA by Minimap2 software (version 2.17.r941, Broad Institute, Cambridge, MA, USA) using the human genome reference sequence Hg38). Sequencing data multi-sequence alignment and identification of pathogenic microorganisms sequencing data filtered by data and stripped of host DNA were performed with Centrifuge v1.0.3 (http://www.ccb.jhu.ed

u/software/centrifuge/, Center for Computational Biology, Johns Hopkins University, Baltimore, MD, USA) and non-redundant nucleic acid database at the National Center for Biotechnology Information. In addition to pathogenic microorganisms, original microbial comparison results also contained a large number of background microorganisms. Therefore, it was necessary to establish specific criteria for pathogenic bacteria identification: (1) The samples included in this study were BALF samples obtained by using fiberoptic bronchoscopy, and normal oropharyngeal microorganisms were excluded [13,14] (**Supplementary Table 1**); (2) Irrelevant microorganisms of pulmonary infection were excluded based on literature retrieval; (3) The species coverage ratio (CR) of bacteria, viruses, and parasites was 10 times higher than those of other microorganisms; That of fungi was more than five times higher; For *M. tuberculosis*, because the extracted DNA content was low, only one sequence was detected to be determined as positive [15,16]; (4) Microorganisms with sequences of bacteria (except tuberculosis), fungi, and viruses less than three were excluded.

The R statistical software (version 4.1.2, R Development Core Team, The R Foundation for Statistical Computing, Vienna, Austria) was used to process the data, and counting data were expressed as a rate (%). χ^2 (Chi-square) test was used for pairwise comparison, and $p < 0.05$ was considered statistically significant. Visualization of co-infected pathogens was analyzed using the UpsetR package in R statistical software.

qPCR Detection

As for results with inconsistent detection by nanopore sequencing and conventional culture, we performed qPCR for validation. DNA of samples was extracted as mentioned above. The qPCR reaction system was prepared under the instructions of the FastKing qPCR kit (FP313, TIANGEN, Beijing, China). Next, qRT-PCR was performed on a 7500 fluorescence quantifier (4351104, Applied Biosystems, Foster City, CA, USA), and the reaction conditions were set as follows: pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 30 s, with a total of 40 cycles. Universal primers 27F/1492R (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R: 5′-TACGGCTACCTTGTTACGACTT-3′) and ITS1/4 (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′; ITS4: 5′- TCCTCCGCTTATTGATATGC-3′) were used for bacterial identification and fungal identification, respectively. The primers used to detect mycoplasma were: forward: 5′-GGCGAATGGGTGAGTAACACG-3′; reverse: 5′-CGGATA ACGCTTGCGACCTATG-3′.

Sputum Culture, TB-DNA, and Xpert MTB/RIF Detection

A sputum sample was collected from all 70 participants. Four times the volume of 4% NaOH was added to the sputum sample and allowed to stand at room temperature for 30 min. Next, 200 μL of the mixture was inoculated to blood agar (024070, Huankai Biology, Guangzhou, China), MacConkey agar (M7408, Millipore Corporation, Billerica, MA, USA), and chocolate agar (LA0040, Solarbio Life Sciences, Beijing, China) mediums, respectively. The samples were considered positive once the pathogenic bacteria grew, which were subsequently used for TB-DNA and Xpert MTB/RIF detection as per the instructions of the TB nucleic acid detection kit (PCR fluorescence probe method) (FS-P4827, Xuanya Biological Technology Co., Ltd., Shanghai, China) and Xpert MTB/RIF assay kit (GXMTB/RIF, Cepheid Innovation, Sunnyvale, CA, USA).

Treatment Strategy Changes after Nanopore Sequencing

Based on the Official Clinical Practice Guideline of The American Thoracic Society and the Infectious Diseases Society of America (ATS/IDSA), it is more effective and safer to use PSI, which is used in this study, to guide the initial CAP treatment than CURB-65 [11]. The sequencing results and the baseline PSI scores were referred to guide the medication administration to LRTI patients, and the treatment outcome of patients was also reported. The clinical medication was based on the latest version of the *Guidelines for the Diagnosis and Treatment of Community-Acquired Pneumonia*. If a patient had tuberculosis, the medication strategy followed the latest edition of the *Guidelines for Prevention and Treatment of Tuberculosis*. Comprehensive evaluation of the therapeutic progress of patients included clinical symptoms (fever, cough, and sputum), imaging, levels of infection indicators (procalcitonin, C-reactive protein, etc.), and blood indicators. No new PSI score was assigned to patients after treatment.

Results

Characteristics of Patients

The clinical characteristics of 70 patients with LRTI are presented (**Supplementary Table 2**). Forty-two patients (60%) had CAP; two had HAP (3%); twelve had bronchiectasis symptoms (17%); four had emphysema and four had pleural effusion. Two additional cases presented with symptoms of both emphysema and bronchiectasis, three had acute exacerbation of chronic obstructive pulmonary disease, and one had lung abscess. Before the collection of BALF, 88.6% of the cases had been treated with different drugs according to specific clinical symptoms.

Table 1. Detection rate of nanopore sequencing and culture method in BALF specimens from 70 patients with LRTI.

	Infection		Non-infection	Positive rate
	Co-infection	Single infection		
Nanopore sequencing	29 (41.4%)	30 (42.9%)	11 (15.7%)	59 (84.3%)
Culture method	3 (4.3%)	15 (21.4%)	52 (74.3%)	18 (25.7%)
z value				6.71
p value				<0.0001

Diagnostic Performance of BALF Pure Microbial Culture

Eighteen specimens were cultured for pathogens (25.71%), including bacteria (13/18, 72%) and fungi (5/18, 28%) (Fig. 2A). Two out of five samples showed single infections of *Candida albicans* and *Candida tropicalis*, respectively. Three BALF specimens were co-infected with both bacteria and fungi (3/18, 17%), including *Pseudomonas aeruginosa* and *C. albicans*, *P. aeruginosa*, *C. tropicalis*, and *P. aeruginosa* and *Aspergillus fumigatus* co-infection.

A total of 10 pathogens were cultured in 18 BALF specimens with seven bacteria and three fungi. *S. aureus* was identified in five samples, *P. aeruginosa* in four samples, *K. pneumoniae* also in 4 samples, and *Streptococcus agalactiae*, *S. pneumoniae*, and *Klebsiella aerogenes* in two samples. Only three fungi, *C. albicans* (2 cases), *C. tropicalis* (1 case), and *A. fumigatus* (1 case) were identified (Fig. 2B).

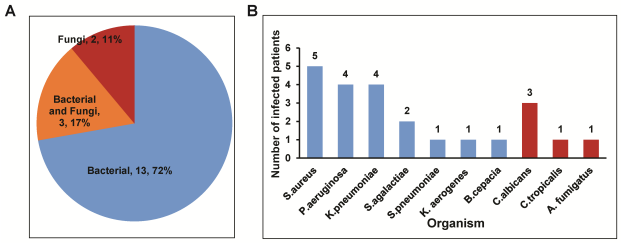

Fig. 2. Classification of pathogens by BALF culture detection. (A) Pathogen diagnosis of 70 BALF samples by traditional culture detection. (B) Species of the detected pathogens. The blue and red columns indicate bacteria and fungi, respectively.

Diagnostic Performance of Third-Generation Metagenomic Sequencing in BALF

Pathogens were detected in 59 BALF samples (84.3%) from 70 samples using third-generation metagenomic sequencing. One pathogen was detected in 30 samples (42.9%). Two or more pathogens were detected in 29 samples (co-infection, 41.4%) (Table 1). The third-generation metagenomic technology presented a higher detection efficiency than the traditional microbial pure culture.

Among the detected 59 BALF cases, a total of 31 pathogens were identified using nanopore sequencing, of which 50% were bacteria, 17% were fungi, 32% were viruses, and 1% were *M. pneumoniae* (Fig. 3A). Nineteen common pulmonary infection bacteria were identified via nanopore sequencing, namely *S. aureus*, *Haemophilus influenzae*, *K. pneumoniae*, and *M. tuberculosis* (Fig. 3B). A majority of detected fungal pathogens were *Candida* and *Aspergillus*. Four of the most frequently isolated Candida were *C. albicans*, *C. tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Aspergillus*, mostly *Aspergillus flavus* and *Aspergillus niger*. Most isolated viral pathogens were human herpes virus type 7 (HHV 7) accounting for 16%, Epstein-Barr virus (EBV) accounting for 9%, HHV 6 accounting for 4%, parvovirus (2%), and bocavirus (1%). Twenty-six virus-infected samples were isolated, indicating that nanopore sequencing had high detection sensitivity for virus-infected patients, which contributed to the clinical detection of virus-infected patients. Nanopore sequencing further isolated one case with *M. pneumoniae* infection.

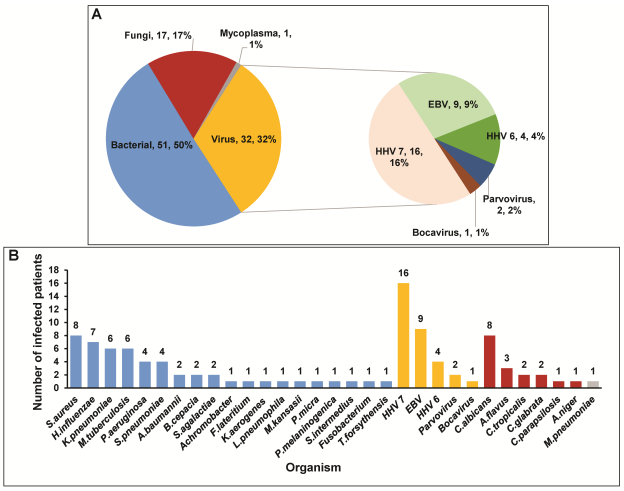

Fig. 3. Classification of pathogens detected by BALF nanopore metagenomic sequencing. (A) Microbial classification of 59 infected samples. (B) The detailed species of the detected pathogens. The blue, yellow, red, and grey columns indicate bacteria, viruses, fungi, and mycoplasma, respectively.

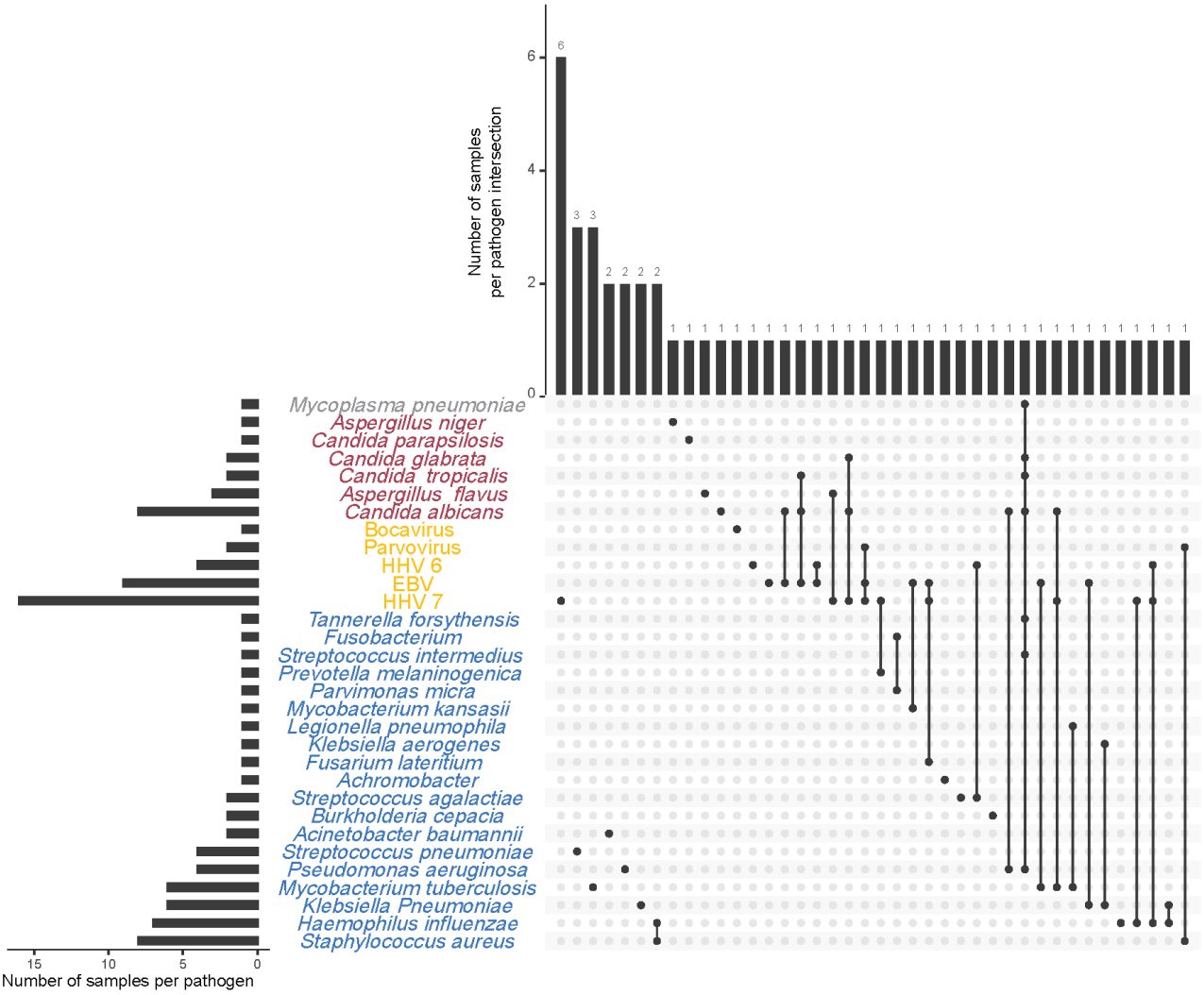

Fig. 4. Pathogen spectrum and co-infection distribution detected by nanopore sequencing.

Fifty-nine infected specimens were identified based on the third-generation nanopore metagenomic sequencing technology. Thirty were single infections, and twenty-nine were co-infected with two or more pathogens. Bacterial and viral infections were the most common type of co-infection (Fig. 4).

Performance Comparison of Nanopore Sequencing and Traditional Microbial Culture

Of the 70 LRTI cases, only 18 (25.7%) infected specimens were positive in the BALF culture. Among them, 56 samples were positive and 14 were negative by qPCR molecular detection, while 59 positive samples and 11 negative samples were detected using the nanopore sequencing technology. The consistency of nanopore sequencing results compared with those of culture, qPCR, and culture plus qPCR were 44.4%, 71.4%, and 70.0%, respectively (Table 2). Taking the composite reference criteria (bacterial culture plus qPCR) as the standard, the third-generation metagenomic detection platform based on nanopore sequencing technology had a sensitivity of 70% and a specificity of 91.7%.

Efficacy of Nanopore Sequencing Technology in Tuberculosis Diagnosis

Tuberculosis is a chronic infectious disease caused by *M. tuberculosis*, which causes pulmonary tuberculosis by invading multiple organs as well as lung involvement. Typically, excretory patients are important sources of infection. Clinical manifestations, imaging examinations, laboratory tests (sputum smears and bacterial culture), and molecular diagnosis are used to identify and diagnose pathogenic bacteria. Among the enrolled 70 clinical cases, a total of 18 patients with pulmonary tuberculosis were diagnosed using Xpert MTB/RIF, sputum TB-DNA, qPCR, imaging examinations, and clinical manifestations, accounting for over 1/4, indicating a high incidence of pulmonary tuberculosis in clinical lung diseases. The positive detection rate of spu-

Table 2. Comparison of nanopore third-generation metagenomic sequencing, conventional culture, qPCR detection, and combined qPCR plus conventional culture.

Outcome (n = 70)	Nanopore sequence (Positive)	Nanopore sequence (Negative)	Agreement (%)
Positive by BALF culture (n = 18)	8	10	44.4
Negative by BALF culture (n = 52)	22	30	57.7
Positive by qPCR (n = 56)	40	16	71.4
Negative by qPCR (n = 14)	11	3	21.4
Positive by qPCR plus culture (n = 58)	49	9	70.0
Negative by qPCR plus culture (n = 12)	1	11	91.7

tum was compared using four different methods: TB-DNA detection (sputum diagnosis criteria: >500 CFU/mL), pure culture of *M. tuberculosis*, qPCR detection, and nanopore sequencing (Fig. 5). The detection rate of *M. tuberculosis* in 18 BALF specimens using pure culture was zero. The detection rate of *M. tuberculosis* was increased to 33.3% using nanopore sequencing (*M. tuberculosis* was isolated from 6 samples). The positive detection rates of BALF qPCR amplification based on *M. tuberculosis* and sputum TB-DNA detection were 27.8% and 55.6%, respectively. Compared with the targeted qPCR amplification technology, nanopore sequencing had a higher positive rate. However, compared with the detection of sputum TB-DNA, the detection rate of nanopore sequencing was lower.

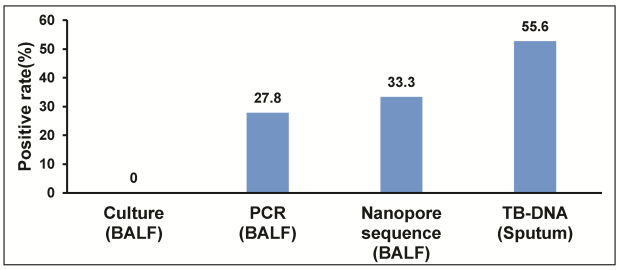

Fig. 5. Positive detection rate of different detection methods in clinical tuberculosis (n = 18). BALF samples were considered positive when the bacteria were identified as *M. tuberculosis* in traditional culture method, *M. tuberculosis* DNA was detected in qPCR detection, and an *M. tuberculosis* sequence was detected in nanopore sequencing; sputum samples were considered positive when *M. tuberculosis* DNA was detected in TB-DNA detection.

Influence of Nanopore Sequencing Technology Detection Results on the Clinical Treatment of Patients with Pulmonary Infection

Among the 70 enrolled patients with LRTI, the clinical treatment protocol for 52 cases (74.3%) was guided by the test results of nanopore sequencing for medication reference (**Supplementary Table 3**). The remaining 17 cases did not refer. One case failed in obtaining the medication information. Of these 52 patients, 31 changed their treatment strategy as per the nanopore sequencing results, 21 supported the previous management, and a total of 47 (90%) patients ultimately achieved clinical improvement.

The medication of the 70 patients with LRTI was analyzed as per PSI grades. Among 28 cases with PSI grade I, 64.3% (18/28) referred to the nanopore sequencing results for medication reference. Ten of them changed the clinical medication regimen, and the other eight supported the previous regimen. Among 26 patients with PSI grade II, 76.9% (20/26) of them referred to the results of nanopore sequencing for medication reference. Twelve changed their medication regimen, while the other eight patients supported the current regimen. There were nine and seven patients with LRTI at PSI grade III and IV, respectively. The results of nanopore sequencing were instructive for all grade III patients. Six changed their medication patterns after the sequencing, while three supported the current medication regimen. Five patients with PSI grade IV referred to the results of nanopore detection and changed the clinical medication regimen, as shown in Fig. 6A.

We then analyzed the 47 patients who were referred to nanopore sequencing results to guide clinical medication with different PSI grades and finally achieved clinical improvement. Clinical outcomes of 18 patients with PSI I LRTI after 14 days of medication indicated significant improvement for 88.9% of the patients (16/18) (Fig. 6B). The remaining two had no improvement. One was infected with pulmonary tuberculosis and was receiving long-term anti-tuberculosis treatment. A total of 90% (18/20) of patients with PSI grade II LRTI improved significantly after 14 days treatment. Only two patients had no improvement after treatment. This included one case of tuberculosis caused by the non-tuberculous mycobacterium complex. All nine patients with PSI grade III LRTI improved after treatment referred to the results of nanopore sequencing. Five patients with PSI IV LRTI significantly improved after 14 days of medication. Only one patient with *C. albicans* infection was still under treatment due to the high degree of infection.

Discussion

For infectious diseases, especially in critically ill infected patients, it is very important to clarify the pathogen.

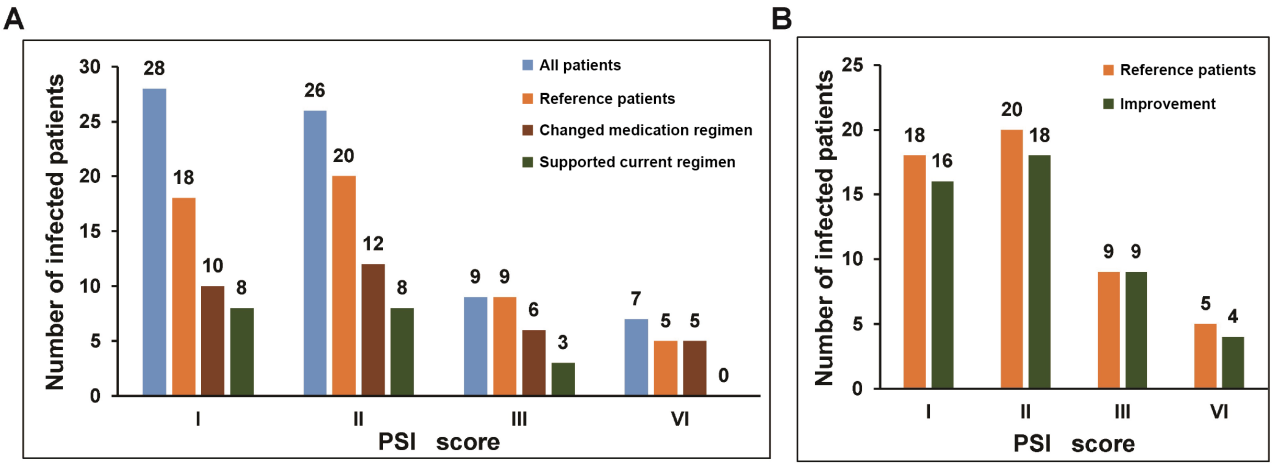

Fig. 6. The effect of nanopore sequencing on patients with LRTI of different PSI scores. (A) The number of patients who changed their medication regimen based on nanopore sequencing results. (B) The improvement of patients after changing the medication regimen.

The establishment of a rapid, accurate pathogen diagnosis method exerts a vital guiding role in the rational use of anti-infective drugs. Traditional bacterial and fungal culture techniques are time-consuming. The positive rate of microbial culture is limited by methodology and antibiotic management. Predominant bacteria and fungi barely grow under conventional culture conditions [17]. As the current high-throughput sequencing technology advances, second-generation metagenomic sequencing (mainly Illumina sequencing platform) has been widely used in the clinical diagnosis of infectious pathogens. The results can be applied as guidance for the rational use of antibiotics in clinical practice, improving clinical efficacy and patient prognosis [18]. Despite second-generation sequencing having high sensitivity and accuracy for microbial detection, it is limited by its experimental principle and takes two days for results. Moreover, it is difficult to differentiate some strains with high homology. Based on literature reports, third-generation metagenomic nanopore sequencing can achieve rapid pathogenic identification of microorganisms in samples in as little as six hours [7,19]. Additionally, it also has the technical advantage of long read length, which can not only accelerate pathogen detection but also improve the specificity of the identification of infectious pathogens. Meanwhile, nanopore sequencers are flexible and convenient in terms of throughput and size, making them more suitable for clinical microbiology laboratories [20]. Unfortunately, due to the insufficient accuracy of base reading before the application of nanopore sequencing technology [9,10] and the advantages and popularity of second-generation metagenomic sequencing, nanopore sequencing technology to detect infectious pathogens in clinical application and research are relatively insufficient, and the current study may serve as a good supplement and exploration in this field.

In 2021, Gu *et al.* [21] performed metagenomic sequencing of cell-free DNA in body fluid samples using second-generation sequencing (based on Illumina platform) and third-generation sequencing (based on Nanopore platform) technologies. The results revealed that the two sequencing technologies are comparable in sensitivity and specificity for the detection of bacteria and fungi. When using clinical standard methods (culture plus PCR) as a reference, the sensitivity of third-generation sequencing technology for bacterial and fungal detection is 75% and 81.4%, respectively, and the specificity is 90.9% and 100.0%, respectively [22]. When using composite criteria such as the independent judgment conclusions of clinical experts or the test results of other sample types as a reference, the positive and negative consistency rates of the third-generation sequencing are 81.0% and 93%, respectively, indicating better detection ability for unknown pathogens. Pearman *et al.* [23] have compared the accuracy of second-generation sequencing (based on Illumina platform) and third-generation sequencing (based on nanopore and PacBio) in classifying pathogens (bacteria and fungi), animals, and plants, using recall rate and classification accuracy rate as the standard. The results have revealed that although third-generation sequencing has a relatively high error rate in base reading compared with second-generation sequencing, the long read length of third-generation sequencing can make up for this disadvantage. Then, for the classification of pathogens (bacteria and fungi), animals, and plants, sequencing read length is more important than accuracy. Sanderson *et al.* [6] have applied second- and third-generation sequencing platforms to identify pathogenic bacteria in the acoustic degradation fluid of nine patients with artificial joint infection. The consistency between the second and third-generation sequencing and culture results is 100%. However, third-generation sequencing indicated better subspecies resolution in one of the samples. Charalampous *et al.* [7] have

performed a retrospective study of bacterial identification in BALF collected from LRTI patients using nanopore sequencing technology, and the results have revealed the sensitivity and specificity of nanopore sequencing technology are 96.6% and 41.7%, respectively as compared to the culture method. However, after qPCR validation and pathogen-specific genetic analysis, the sensitivity and specificity of nanopore sequencing are both improved to 100%. Meanwhile, nanopore sequencing can also accurately detect antibiotic resistance genes. Another exciting use of this technology is its ability to detect viruses. Compared with the traditional detection method, the sensitivity and specificity of nanopore sequencing for the detection of influenza virus are 83% and 100%, respectively [24], which indicates that the nanopore technique has potential application in the diagnosis and genetic analysis of influenza virus and other respiratory viruses.

As a novel tool, nanopore sequencing technology has greatly improved the etiological diagnosis of LRTI. Among the 70 BALF cases in the current study, the positive rate of pathogen detection using nanopore sequencing was 84.3%, much higher than 25.7% of conventional culture. Nanopore sequencing presented the advantage of high sensitivity and based on the composite reference method of culture and qPCR detection results, nanopore sequencing had a sensitivity of 70% and a specificity of 91.7% for BALF pathogen detection with higher accuracy. Among the 59 positive cases using nanopore sequencing, 31 pathogens were identified, covering bacteria, fungi, viruses, and *M. pneumoniae*. The 18 positive pathogen profile cases using culture included only 13 cases of bacteria and five cases of fungi, and the Xpert MTB/RIF detection rate was 0. It can be seen that the comprehensiveness of nanopore detection of infectious pathogens was also much higher than that of conventional culture. As stated in the introduction, traditional culture lacks accuracy in pathogen detection. We speculated that this may have affected the 62 patients who had been on antibiotics before the specimens were collected, possibly placing the bacteria in a suppressed state. In addition, we used blood agar, MacConkey agar, and chocolate agar to culture the bacteria for 24 h, but some of the bacteria need more nutrients and time to grow.

By comparison, the detection of *M. tuberculosis* samples (**Supplementary Table 4**) indicated that patients with false negative nanopore sequencing results had lower concentrations of TB-DNA in their sputum. For clinical patients in this part, the positive detection rate of patients can be effectively improved by increasing the data volume of nanopore sequencing detection. Unfortunately, the Xpert MTB/RIF data, the gold standard for the detection of *M. tuberculosis* in this study, were incomplete, and most samples were not detected. Therefore, comprehensive data for nanopore sequencing to be compared were not available. The results of 4 in 6 tested cases were consistent (2 positive and 2 negative, 67%). Although the epidemic trend of tuberculosis in China has declined, the unbalanced control of the epidemic in various regions is still a prominent problem for public health. Our data indicate that it is possible that metagenomic detection based on nanopore sequencing technology will contribute to the early diagnosis and early treatment of pulmonary tuberculosis in the future, and provide a better scientific and technical basis for the elimination of tuberculosis by 2035.

Among the 70 enrolled patients with LRTI, the clinical treatment plan for medication for 52 cases was guided by the test results of nanopore sequencing. Forty-seven achieved clinical benefit, a rate of 90%, indicating that nanopore sequencing for pathogen diagnosis has great clinical application value. In particular, it provided a novel and effective technical means for LRTI patients when pathogens failed to be rapidly detected using traditional culture. A control group without reference to the results of nanopore sequencing can be set, and it is believed to better reflect the clinical significance of nanopore sequencing technology.

This study also has limitations. First, The sample size (N = 70) is small. A control group without reference to the results of nanopore sequencing can be set, and it is believed to better reflect the clinical significance of nanopore sequencing technology. Second, though we excluded the results unassociated with a lung infection, not all the detected pathogens would be of clinical significance. We have to combine the specific clinical symptoms of each patient and take the sequencing results as guidance for medication. Third, in addition to the improvement of specificity and accuracy, the long-read length advantage of third-generation nanopore sequencing also plays an essential role in the detection of structural variation of drug resistance genes, while it is not covered in this study. In the future, the drug resistance application of microbial infection in respiratory diseases will be more extensive. The corresponding authors have made great efforts on the drug resistance database, and they are committed to making a localized drug resistance database available for matching the microbial drug resistance genotype with phenotype. Subsequent studies will highlight the integration of pathogen identification and drug resistance.

Conclusions

When it comes to detecting pathogens, the third-generation sequencing technology outperforms traditional culture methods by having a higher detection rate. Nonetheless, this advantage diminishes when using culture plus qPCR as the reference standard. Nevertheless, given the faster detection speed of sequencing technology, we believe that it can provide earlier assistance for patient treatment. Overall, we conclude that third-generation sequencing technology holds significant potential for pathogen detection in LRTI and remains an attractive option for future diagnosis and treatment strategies.

Availability of Data and Materials

The data used to support the findings of this study are included within the article.

Author Contributions

Conceptualization, ZO, HD and YS; methodology, WL, JX, CL and JL; software, YH and SZ; validation, JX and SZ; analysis, YH, JX and JL; writing—original draft preparation, WL, YH and JX; writing—review and editing, ZO, HD; visualization, CL; project administration, ZO, HD and YS. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study involving human participants was reviewed and approved by the Ethics Review Committee of NanFang Hospital of Southern Medical University (No. NFEC-2021-018). The participants provided their written informed consent to participate in this study.

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Conflict of Interest

The authors declare no conflict of interest. YH, JL and ZO are employees of Dian Diagnostics Group Co., Ltd., they declare no conflict of interest (even superficial conflict) with the company, its shareholder and its customers.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202335176.34.

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