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Clinical Impact of Metagenomic Next-Generation Sequencing of Bronchoalveolar Lavage in the Diagnosis and Management of Pneumonia

A Multicenter Prospective Observational Study

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Rapid and accurate pathogen identification is necessary for appropriate treatment of pneumonia. Here, we describe the use of shotgun metagenomic next-generation sequencing (mNGS) of bronchoalveolar lavage for pathogen identification in pneumonia in a large-scale multicenter prospective study with 159 patients enrolled. We compared the results of mNGS with standard methods including culture, staining, and targeted PCR, and evaluated the clinical impact of mNGS. A positive impact was defined by a definitive diagnosis made using the mNGS results, or change of management because of the mNGS results, leading to a favorable clinical outcome. Overall, mNGS identified more organisms than standard methods (117 versus 72), detected 17 pathogens that consistently were missed in all cases by standard methods, and had an overall positive clinical impact in 40.3% (64 of 159) of cases. mNGS was especially useful in identification of fastidious and atypical organisms causing pneumonia, contributing to detection of definitive pathogens in 45 (28.3%) cases in which standard results were either negative or insufficient. mNGS also helped reassure antibiotic de-escalation in 19 (11.9%) cases. Overall, mNGS led to a change of treatment in 59 (37.1%) cases, including antibiotic de-escalation in 40 (25.2%) cases. This study showed the significant value of mNGS of bronchoalveolar lavage for improving the diagnosis of pneumonia and contributing to better patient care. *(J Mol Diagn 2021, ■: 1–10; https://doi.org/10.1016/j.jmoldx.2021.06.007)*

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Pneumonia is a global health concern associated with high morbidity and mortality as well as increased hospital admissions rates.[1] Diagnosis typically is made based on a combination of clinical manifestation, radiographic features, microbiological culture, and/or molecular results. Radiographic features, although indicative of a pneumonia, do not provide pathogen identification, which is necessary to guide treatment.[2] One study found that a pathogen was detected in only 38% of community-acquired pneumonia cases in the United States,[3] emphasizing the importance of more sensitive and rapid diagnostics tools. In the case of atypical, fungal, or viral pneumonia, organisms are unable to be cultured by routine methodologies, are slow-growing, or require specific molecular assays, which are not always widely available or standardized.[4] These organisms include atypical bacterial pathogens such as *Chlamydia psittaci*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, and *Legionella* species, which accounted for roughly 15% of community-acquired pneumonia in one study,[4] and respiratory viruses such as human rhinovirus and influenza viruses, which accounted for up to 27% of community-acquired pneumonia in another study.[3] Among immunocompromised patients, *Aspergillus fumigatus*, *Pneumocystis jirovecii*, and cytomegalovirus (CMV) frequently are associated with lower-respiratory infections.[5] These pathogens pose challenges with diagnostics. Molds, including *Aspergillus* species, yield low recovery from primary patient specimens in culture[6] while CMV requires a targeted PCR and thus a degree of suspicion. The conventional method for the detection of *P. jirovecii* relies mainly on direct staining, which lacks sensitivity, although the molecular assay for *P. jirovecii* is not widely available.[7] Because of the inability to rapidly and accurately diagnose and differentiate between viral, bacterial, and fungal agents of respiratory infections, antibiotic overuse remains a persistent problem.[8,9]

Metagenomics next-generation sequencing (mNGS) is an unbiased molecular approach for detecting all DNA and/or RNA content from clinical samples, allowing for identification of bacteria, viruses, fungi, and parasites simultaneously.[10] mNGS has aided in the diagnosis of respiratory infections in several case reports and studies,[11–16] but its utility has not been shown specifically for pneumonia by large clinical studies. Here, we describe the use of mNGS of bronchoalveolar lavage (BAL) for the diagnosis of pneumonia across 12 hospitals in Zhejiang Province, China. BAL samples were collected from patients with symptoms and chest X-rays consistent with pneumonia and were sent for standard microbiological tests (including culture, staining, and several targeted molecular assays) and mNGS, respectively. The goal of this study was to determine the clinical impact of mNGS results on both the diagnosis and management of pneumonia.

Materials and Methods

Patient Enrollment

A total of 159 patients with pulmonary infections were enrolled in this study from January 2018 to December 2019 from multiple clinical centers (Table 1), led by the Sir Run Run Shaw Hospital, Zhejiang University School of Medicine. The following inclusion criteria were used: i) the patients were not limited by age or sex; ii) the initial clinical diagnosis was pneumonia based on positive radiographic findings (chest X-ray or lung computed tomography) and clinical presentations including new onset of cough, exacerbation of original cough, sputum production, fever, or shortness of breath; iii) the patients could tolerate bronchoscopy and had adequate BAL collected; and iv) the patients could read the informed consent, understood and were willing to cooperate with the research plan, and signed relevant documents.

Patients who tested positive for the respiratory RNA viruses including influenza A, influenza B, parainfluenza viruses 1 to 4, and respiratory syncytial virus were excluded from this study because these patients (after being screened at the fever clinics) typically were admitted to a special ward with enhanced isolation precautions designated to treat patients infected with highly communicable respiratory viruses.

This research was approved by the ethics committee of Sir Run Run Shaw Hospital, Zhejiang University School of Medicine (Hangzhou, Zhejiang, China) (approval 20191025-6). All patients or family members signed the informed consent.

Samples and Laboratory Testing

All patients had bronchoscopy performed. The BAL was collected and aliquoted for both standard laboratory testing and mNGS (Supplemental Figure S1). The standard diagnostic methods for BAL include bacterial, mycobacterial, and fungal cultures; acid-fast bacilli smear stain for mycobacteria; modified acid-fast bacilli stain for *Nocardia*; direct fluorescence antibody stain for *P. jirovecii*; calcofluor white stain for fungi; galactomannan antigen test; and PCR assays for *Mycobacterium tuberculosis* complex (GeneXpert, Cepheid), *M. pneumoniae*, *C. pneumoniae*, and CMV (Liferiver Biotechnology, Shanghai, China). In addition, 1,3-β-D-glucan, galactomannan, and *Cryptococcus* antigen tests also were performed on the serum samples.

mNGS Testing

BAL samples were collected and immediately transported on dry ice to a commercial laboratory (IngeniGen XunMinKang Laboratory, Zhejiang, China) for mNGS testing within 8 hours after collection. Briefly, DNA was extracted from 300 μL BAL and the sequencing library was prepared using the Total Nucleic Acid Extraction Kit and the mNGS-DNA Library Prep Kit (IngeniGen XunMinKang Biotechnology). Sequencing was performed using the 75-bp paired-end protocol on the Illumina Nextseq550 platform. At least 2.5 million reads (75 bp) were obtained from each sample after sequencing. The sequence data were analyzed by IngeniSeq-MG version 1.0 mNGS software (IngeniGen

XunMinKang Biotechnology), which contains a proprietary curated database consisting of more than 20,000 microbial reference genomes. The detailed methods regarding the wet-lab and bioinformatics have been described previously.[13] Most mNGS results were returned within 36 hours.

Data Analysis and Quality Control

Several quality control measures were used to ensure the validity of the mNGS results. First, a negative control was included in each mNGS run to detect background microbial DNA contaminants. A true-positive result was valid only when the sequence reads exceeded 10 times more than the corresponding reads in the negative control. Second, to avoid a false-positive result caused by cross-contamination or "spill over," a true-positive result was valid only when the sequence reads of a species in a sample exceeded 10% of the total reads of the same species in all samples in the same run. Third, the internal control (a unique marine bacteria spiked in each sample) should have more than 100 reads detected for a negative result to be valid. Fourth, the "environmental" species (predetermined by the IngeniSeq-MG version 1.0 mNGS software) that were present with more than 10% frequency in the negative controls over the past 100 runs were considered as contaminants and filtered out from the final results. Fifth, the "normal flora" species (normally colonizing in the human respiratory tract, predetermined by the IngeniSeq-MG version 1.0 mNGS software) also were filtered out as contaminants if their reads were lower than a specific cut-off value built into the software. The specific thresholds for filtering out normal flora were determined by a metadata analysis of more than 5000 BAL samples (proprietary data of IngeniGen XMK Biotechnology).

Determination of Clinical Impact

A positive impact was defined by a definitive diagnosis made using the mNGS results, or change of management as a result of the mNGS results, leading to a favorable clinical outcome. A negative impact was defined by a wrong diagnosis made using the mNGS results that lead to unnecessary or suboptimal treatment.

Results

Patient Summary

In total, there were 102 males and 57 females across 12 hospitals, with ages ranging from 1 to 86 years (mean, 50.3 years; SD, 19.1 years) (Table 1). Thirteen (8.2%) patients were younger than age 18 years. All patients had radiographic findings consistent with a pulmonary infection (Supplemental Table S1), with the most common symptoms being fever (67.3%), cough (61.0%), and shortness of breath (22.6%) (Table 1). Approximately two thirds of the patients (108 of 159) had certain underlying conditions including diabetes ($n = 17$); solid tumor ($n = 17$), of which 13 were lung cancer; hematological malignancy ($n = 9$), of whom 6 received a stem cell transplant; and chronic lung diseases ($n = 27$) such as bronchiectasis, asthma, chronic obstructive lung diseases, pulmonary fibrosis, interstitial lung diseases, and pulmonary tuberculosis. Other underlying conditions included hypertension, coronary artery diseases, chronic hepatitis B, chronic liver diseases, renal dysfunction, and so forth. Only one patient had a previous diagnosis of HIV infection. Many patients had multiple underlying conditions. The majority of the patients (153 of 159; 96.2%) were cured (80 of 159; 50.3%) or clinically improved (74 of 159; 46.5%). Three patients did not achieve clinical improvement and two patients died of comorbidity. The detailed clinical information is described in Supplemental Table S1.

Summary of mNGS Quality Matrices

On average, 14,349,276 reads (75 bp) were acquired for each sample (minimum, 2,865,622 reads; maximum, 54,835,140 reads; median, 12,423,268 reads). The majority (152 of 159; 95.6%) of the samples had more than 5 million reads, and 66.0% (105 of 159) of samples had more than 10 million reads. Approximately half (78 of 159) of the samples had sequence reads between 10 and 20 million, and 86.2% (137 of 159) of samples had sequence reads between 5 and 25 million. After human DNA sequence filtering, 71.1% (113 of 159) of samples had less than 10% nonhuman sequences. The median nonhuman sequence percentage was 1.78 (minimum, 0.56; maximum, 97.24), indicating that most samples had a high percentage of host DNA. The distribution of the total sequence reads and the nonhuman reads are shown in Supplemental Figure S2. The most common normal flora detected by mNGS included *Staphylococcus epidermidis* and *Prevotella melaninogenica*, which were detected in approximately half of the samples. Other frequently detected normal flora included coagulase-negative *Staphylococcus* species, *Streptococcus* species, *Prevotella* species, *Corynebacterium* species, *Fusobacterium nucleatum*, *Neisseria elongata*, *Porphyromonas gingivalis*, *Parvimonas micra*, and many other bacteria typically found in the oral cavity (Supplemental Figure S3). These normal flora species were mostly filtered out and reported only when their sequence reads exceeded a certain threshold built into the software (as described in *Materials and Methods*). The samples were tested in 101 separate runs and all of the environmental microbes considered as contaminants (detected in the 101 negative controls) are summarized in Supplemental Table S2. The top 10 most common contaminants included *Cutibacterium acnes*, *Mycoplasma wenyonii*, *Comamonas testosteroni*, *Acinetobacter johnsonii*, *Acidovorax* species, *Delftia tsuruhatensis*, *Pseudomonas geniculata*, *Pseudomonas fluorescens*, *Moraxella osloensis*, and *Thermus scotoductus*.

Table 1 Enrolled Patient Demographics and Clinical Manifestation

XXX	*n* (%)
Sex	
Male	102 (64.2)
Female	57 (35.8)
Age, years	n (%)
0–18	13 (8.2)
19–30	14 (8.8)
31–50	38 (23.9)
51–65	62 (39.0)
>65	32 (20.1)
Primary clinical symptoms	
Radiographic findings	159 (100)
Fever	107 (67.3)
Cough	97 (61.0)
Shortness of breath	36 (22.6)
Increased sputum production	15 (9.4)
No overt symptoms	10 (6.3)
Chest pain	6 (3.8)
Hemoptysis	4 (2.5)
Fatigue	1 (0.6)
Dizziness	1 (0.6)
Chest congestion	1 (0.6)

The total number of patients enrolled in the study was 159.

Comparison of mNGS Results with Standard Methodology

mNGS and standard results had complete agreement in 85 (53.5%) cases, with negative and positive results in 67 and 18 cases, respectively. Standard methods were unable to identify any organism in 101 of the 159 cases, while mNGS identified the following number of unique organisms per sample: one ($n = 22$), two ($n = 6$), or three or more ($n = 6$) (Table 2). On the contrary, mNGS was negative in 83 cases and, of these cases, standard methods identified one organism ($n = 14$), two organisms ($n = 1$), or three or more organisms ($n = 1$) (Table 2). Overall, mNGS identified more bacterial (89 versus 54), fungal (18 versus 15), and viral (10 versus 3) organisms compared with standard methods (Figure 1). There were 17 and 4 organisms identified only by mNGS or standard methods, respectively (Figure 2). Bacteria that were known to cause pneumonia and identified only by mNGS included *C. psittaci*, *Haemophilus influenzae*, *Legionella pneumophila*, *Mycobacteroides abscessus*, *Mycobacterium avium*, and *Actinomyces* species, and so forth. In addition, mNGS also identified varicella zoster virus ($n = 1$) and adenoviruses ($n = 3$) that were not included in the standard tests (Figure 2). There were only four organisms identified only by standard methods: *Elizabethkingia meningoseptica*, *Providencia stuartii*, *Staphylococcus cohnii*, and *Cryptococcus* (Figure 2). Among these, only *Cryptococcus* was considered a pathogen in this study, however, it was detected only by the serum antigen test in the blood. The likely reasons for the missed species include the following: a very low microbial load in the sample that was below the limit of detection of mNGS, or the species was filtered out as normal flora or environmental contaminants by the software.

When examining the cases in which mNGS was positive and standard methods were negative, the most commonly identified organisms were *P. jirovecii* ($n = 8$ cases), *M. pneumoniae* ($n = 5$ cases), *Streptococcus pneumoniae* ($n = 4$ cases), *Pseudomonas aeruginosa* ($n = 4$ cases), *Klebsiella pneumoniae* ($n = 3$ cases), and *Streptococcus intermedius* ($n = 3$ cases) (Supplemental Table S3). For cases in which mNGS was negative but standard methods identified an organism, the most commonly identified organism was *M. tuberculosis* ($n = 5$ cases) (Supplemental Table S3).

Clinical Impact of mNGS Results on Diagnosis and Treatment

Although all diagnoses in part relied on the clinical picture, mNGS, standard methods, or both mNGS and standard methods were used in 36 (22.6%), 39 (24.5%), and 28 (17.6%) cases for a final diagnosis, respectively (Figure 3). When examining the impact of mNGS results on patient care, mNGS results showed a positive or no impact in 64 (40.3%) and 94 (59.1%) cases, respectively, whereas a negative impact was observed in only one single case (0.6%) (Table 3). In the positive impact cases, positive mNGS results contributed to a definitive diagnosis in 45 cases, and negative mNGS results helped rule out active infection in 19 cases. In the cases without impact, mNGS did not detect any additional pathogen in 86 cases, and its results were deemed contaminants or insignificant in 8 cases (Table 3).

In terms of patient management, mNGS results directly led to a change in treatment (59 of 64 positive impact cases), or a definitive diagnosis that helped continue the empirical treatment (5 of 64 positive impact cases). In the cases in which there was a change in treatment owing to the mNGS results, 40 of 59 of those cases resulted in a de-escalation of treatment (antibiotics were discontinued or changed to a narrower spectrum). Among the 64 cases with positive impact, all except one patient achieved clinical cure or

Table 2 Comparison of Organisms Detected by mNGS and Standard Methods for Each Specimen

		Standard ($n = 68$ organisms)			
		Negative	1	2	3+
mNGS ($n = 118$ organisms)	Negative	67	14	1	1
	1	22	28	3	0
	2	6	7	1	1
	3+	6	0	1	1

mNGS, metagenomics next-generation sequencing.

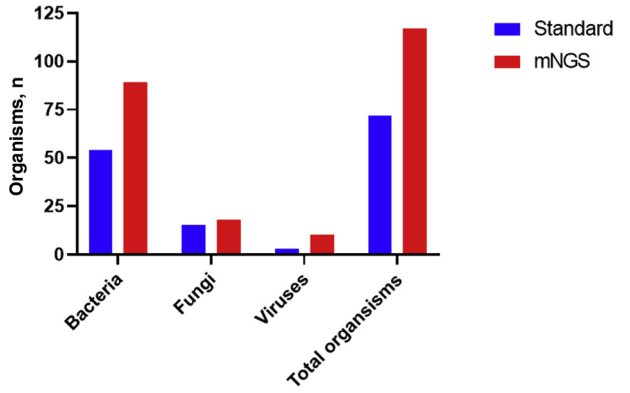

Figure 1 Type of organisms detected by metagenomics next-generation sequencing (mNGS) compared with standard methodologies.

improvement. In one case, mNGS results led to a diagnosis of pneumonia caused by both CMV and *Nocardia*, but the patient died of comorbidity despite appropriate treatment. Notably, mNGS identified multiple types of pathogens requiring different targeted treatments in 9 cases (Table 4). These cases included detection of two to four pathogens encompassing mycobacteria (eg, *M. tuberculosis*, *M. avium*), atypical bacteria (eg, *Legionella*, *Nocardia*), common bacteria (eg, *Staphylococcus aureus*, *Escherichia coli*), fungus (eg, *Aspergillus*, *P. jirovecii*), and virus (eg, CMV), highlighting the complexed etiologies of pneumonia in certain cases and the strength of mNGS, which is capable of detecting all of them in one test.

Discussion

Previous studies have shown the utility of sequencing for pathogen detection directly from fixed and fresh lung tissues,[12,17] as well as a diagnosis of pneumonia in mechanically ventilated patients.[18] Here, we describe the clinical utility of mNGS on BAL for the diagnosis and management of pneumonia across multiple health care systems in China, where routine molecular assays are limited. Our study highlights key opportunities in which mNGS provided species-level identification of a respiratory pathogen, serving as the sole mechanism for diagnosis or supplementing standard results. One such area was for infections caused by common pathogens that were not recovered in culture. For example, *S. pneumoniae*, a common cause of bacterial pneumonia,[2] was detected by mNGS in four of eight pediatric patients younger than age 9, leading to a definitive diagnosis; these cases were missed by standard culture. Other pathogens that had low yield by regular culture but were detected by mNGS in this study include *Haemophilus influenzae*, *Actinomyces* species, *Nocardia* species, and anaerobic bacteria.

Another advantage of mNGS includes the ability to detect respiratory viruses that currently are not screened for routinely in patients with pneumonia in China, including human adenovirus B55 and varicella-zoster virus. In these cases, mNGS led to a definitive diagnosis and had a positive impact on patient care. Most importantly, mNGS overcomes a critical obstacle that has long plagued traditional culture

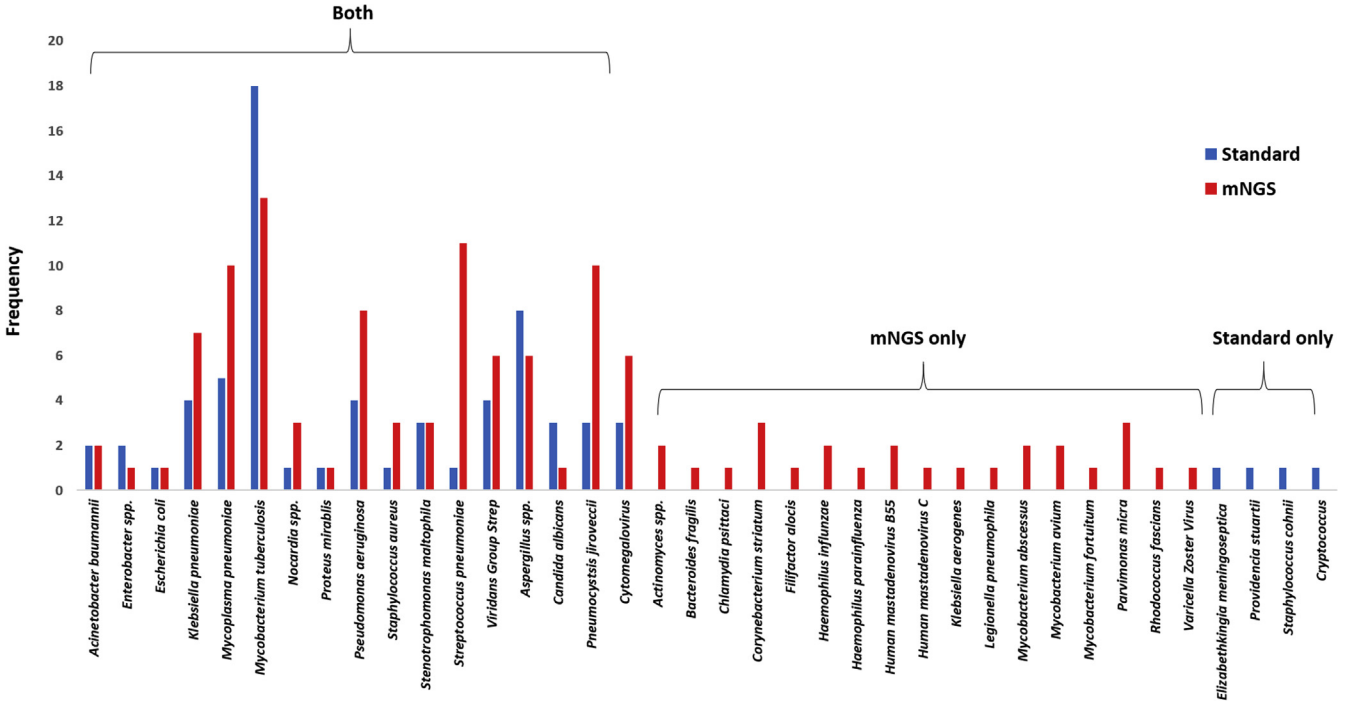

Figure 2 Breakdown of organisms identified by both metagenomics next-generation sequencing (mNGS) and standard methods, mNGS only, and standard methods only.

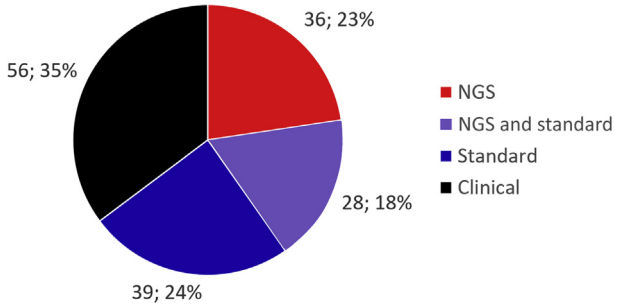

Figure 3 Methodology used to guide treatment and diagnosis (n = 159 cases). NGS, next-generation sequencing.

methodologies: the ability to diagnose atypical pneumonias in which organisms are unable to be recovered in normal culture conditions and routine molecular assays are limited. In this study, atypical bacteria identified by mNGS that were missed by standard methods included *M. pneumonia*e, *L. pneumophila*, and *C. psittaci*. Although there is a PCR test available for *M. pneumoniae*, it was lacking sensitivity in this study and 5 of 10 cases were detected only by mNGS. In the group of patients ages 8 to 17 years in this study, *M. pneumonia* was the most common bacterial organism detected by mNGS (5 of 7 patients), which is a leading cause of atypical pneumonia in children older than age 5 years.[2] However, it is important to point out the lower sensitivity of the *M. pneumoniae* PCR used in this study may be a unique case and may not represent a universal phenomenon. The other atypical pneumonia pathogens, *L. pneumophila* and *C. psittaci*, were identified only by mNGS. *Legionella* is slow-growing bacteria that is responsible for severe community-acquired pneumonia and extrapulmonary manifestations.[4,19] A rapid diagnosis is essential because *Legionella* is resistant to empiric β-lactam therapy.[19] Currently, molecular methods are neither standardized nor widely available and diagnosis relies on slow-growing culture requiring special media or a urinary antigen test that only detects one serogroup.[19] Previously, direct sequencing from respiratory samples has shown promise for both identification of *Legionella* and molecular epidemiological investigations.[20] *C. psittaci* is a zoonotic pathogen that is transmitted from birds to humans and is responsible for psittacosis.[21] It has been implicated in endocarditis, pneumonia, and encephalitis, but infected patients typically present with influenza-like symptoms.[21] This organism is difficult to identify, but NGS has been used previously to diagnosis a case of severe pneumonia and multi-organ failure as a result of *C. psittaci*,[11] further highlighting the role of NGS in diagnosing this organism. It is important to point out that mNGS also detected non-TB Mycobacteria, including *M. avium* and *M. abscessus*, which were missed by standard methods in three cases, leading to a definitive diagnosis, highlighting the strength of mNGS for species-level identification of mycobacteria.

Fungal respiratory infections also are particularly difficult to diagnose by traditional means. mNGS provides accurate species-level identification[17] and has been found to have a high specificity[12] for detection of fungal pathogens in lung specimens compared with histology.[22] Fungal culture is slow and arduous and identification relies on macroscopic and microscopic morphology, which requires a pure isolate with sporulation and distinctive features.[23] Other studies have shown the utility of mNGS in identification of *Aspergillus* and *Coccidioides* in BAL, when clinical microbiological testing was negative.[24] In this study, mNGS (which detected *Aspergillus* in six cases) provided a good complementation to culture (which detected *Aspergillus* in eight cases) to maximize the overall detection of *Aspergillus* in 12 cases (50% improvement), leading to effective antifungal treatment in all cases except for one case in which the *Aspergillus* detected by mNGS was deemed a contamination or colonization owing to inconsistent clinical presentation. Notably, mNGS showed *Aspergillus terreus* in one case that culture missed. This has important clinical significance because *A. terreus* has intrinsic amphotericin B resistance.[25] In addition to the low recovery rate of molds by culture, other fungi, such as *P. jirovecii*, are unable to be cultured. *P. jirovecii* is an opportunistic pathogen that is a major cause of pneumonia and mortality in immunocompromised patients.[26] Diagnosis relies primarily on an insensitive fluorescent antibody test, and, more recently, a *P. jirovecii* PCR, which is not widely available or standardized.[26] Both methods require a high degree of clinical suspicion to even order, leading to missed diagnosis. We found that to be consistent with our study in which mNGS detected an additional eight cases of *P. jirovecii* that standard methods

Table 3 Clinical Impact and Role of mNGS Result

Clinical impact	Role of mNGS result	Treatment changes owing to mNGS
Positive impact (n = 64; 40.3%)	Contributed to definitive diagnosis (n = 45; 28.3%)	Empirical treatment continued (n = 5; 3.1%) Treatment adjusted without de-escalation (n = 19; 11.9%)
	Helped rule out active infection (n = 19; 11.9%)	Antibiotic de-escalated (n = 40; 25.2%)
Negative impact (n = 1; 0.6%)	False-positive result led to incorrect diagnosis (n = 1; 0.6%)	Unnecessary antibiotic treatment
No impact (n = 94; 59.1%)	No additional pathogen detected (n = 86; 54.1%) Results deemed false or insignificant (n = 8; 5.0%)	No changes

mNGS, metagenomics next-generation sequencing.

Table 4 Cases in Which mNGS Identified Multiple Pathogens Requiring Different Treatments

Sample ID	Standard results	mNGS	Final diagnosis	Anti-infective treatment before mNGS	Anti-infective treatment after mNGS	Outcome
BAL20001	CMV-PCR (+), serum 1,3-β-D-glucan (+)	CMV, *Pneumocystis jirovecii*	CMV pneumonia, *Pneumocystis pneumonia*	Voriconazole, oseltamivir, cefoperazone-sulbactam, SMZ-TMP	SMZ-TMP, caspofungin, ganciclovir	Cure
BAL20007	AFB smear (+), TB-PCR (+)	MTB, *Staphylococcus aureus*	Pulmonary tuberculosis, Staphylococcal pneumonia	Cefoperazone-sulbactam	Antituberculosis therapy, linezolid	Improve
BAL20009	Negative	*Klebsiella aerogenes*, *P. jirovecii*	Bacterial pneumonia, *P. pneumonia*	Cefoperazone-sulbactam	Cefoperazone-sulbactam, SMZ-TMP	Cure
BAL20017	Negative	*Escherichia coli*, *P. jirovecii*	*P. pneumonia* and *E. coli* pneumonia	Cefoperazone-sulbactam, moxifloxacin	Cefoperazone-sulbactam, SMZ-TMP	Cure
BAL20019	*Klebsiella pneumoniae*	*Legionella pneumophila*, *K. pneumoniae*	Legionella and *K. pneumoniae*	Imipenem, voriconazole	Imipenem, voriconazole, levofloxacin	Cure
BAL20020	Negative	*Streptococcus pneumoniae*, *P. jirovecii*	Streptococcal pneumonia, *P. pneumonia*	Cefoperazone-sulbactam	Cefoperazone-sulbactam, SMZ-TMP	Cure
BAL20028	Aspergillus species; BAL GM (+); modified AFB stain (+)	Aspergillus species, Nocardia species	Invasive pulmonary aspergillosis, Nocardia pneumonia	Cefoperazone-sulbactam	Voriconazole, SMZ-TMP	Improve
BAL20039	Modified AFB stain positive; serum 1,3-β-D-glucan (+)	Nocardia species, *Mycobacterium avium*, *P. jirovecii*	Pulmonary nocardiosis, *P. pneumonia*	Cefoperazone-sulbactam, voriconazole	SMZ-TMP, caspofungin	Improve
BAL60008	Negative	*S. pneumoniae*, *Haemophilus influenzae*, *P. jirovecii*, CMV	CMV pneumonia, *P. pneumonia*	Imipenem, voriconazole	SMZ-TMP, ganciclovir	Improve

AFB, acid-fast bacilli; BAL, bronchoalveolar lavage; CMV, cytomegalovirus; mNGS, metagenomics next-generation sequencing; MTB, *Mycobacterium tuberculosis*; SMZ-TMP, trimethoprim/sulfamethoxazole.

missed. In our positive impact cases in which mNGS identified multiple pathogens requiring different treatments, six of nine of these cases included *P. jirovecii*, highlighting the positive impact of mNGS on guiding appropriate treatment coverage.

Notably, mNGS missed five cases of *M. tuberculosis*. On further investigation, this likely was owing to an insufficient DNA extraction protocol used by mNGS in this study because it did not include a bead beating step that can help break the cell wall of mycobacteria more thoroughly, serving as a reminder that the optimization of sample preprocessing is essential for pathogen recovery regardless of what downstream molecular method is used. Our study supports the use of both mNGS and standard methods to maximize pathogen recovery and improve the accuracy of diagnosis.

Overall, the use of mNGS had a positive impact in 64 (40.3%) cases, including a definitive diagnosis, identification of additional pathogens, and providing reassurance for antibiotic de-escalation when a patient is clinically improving and lacks signs of an active infection. In addition to enhanced pathogen detection by mNGS, our study also showed the value of negative mNGS results in 19 cases in which standard methods also yielded all negative results and patients were improving clinically without signs of an active infection. In such settings, the negative mNGS result helped confirm the choice to de-escalate antibiotic treatment.

There was only one single case of negative impact, specifically a false-positive mNGS result leading to an incorrect diagnosis and ineffective treatment. In this case, *Proteus mirabilis* was detected by mNGS but the standard test results all were negative. The patient was treated with antibiotics for *P. mirabilis* but did not respond, and, eventually, was diagnosed with cryptogenic organizing pneumonia, a rare interstitial lung disease. In addition, mNGS results were deemed contaminants or normal flora in eight cases, despite a rigorous algorithm built into the bioinformatic software that filtered out potential contaminants.

This study showed that detection of colonization as well as human or environmental contamination can be problematic for mNGS because of its unbiased and wide-spectrum microbial DNA detection.[27–29] Background contamination and colonization are particularly concerning for specimens with lower pathogenic bacterial loads.[30] There are currently no standardized methods for differentiating between contamination, colonization, and true etiological agents when using mNGS technology only. For instance, we identified *P. micra*, which typically is considered normal oral flora, but can cause aspiration pneumonia, endocarditis, and abscesses.[31] To circumvent these issues, negative controls should be included at every step of the sample preparation and sequencing process[32] and stringent bioinformatics thresholds should be established to filter out laboratory contamination and reduce within-run spillover from high positive samples.

In addition, mNGS results also should be evaluated in the context of clinical presentation and concurrent laboratory results, including bacterial and fungal culture, direct smear staining, histology, and serology. However, difficulties arise when gold standard method results conflict with mNGS results. In this study, the clinicians used their clinical judgment to decide whether to act on the gold standard results, mNGS results, or both. This is an imperfect approach, but one that often is relied on when methodologies measure different entities (nucleic acid versus viable organism) with varying sensitivities. Although unavoidable owing to ever-changing technologies, standardization of mNGS methodologies and analysis as well as recognizing the major limitations of mNGS can begin to address these issues.

Transient microbial DNA, representing either live or dead microbes in the specimen source area, also may confound the mNGS results and warrants interpretation with extreme caution when many species are detected.[33] Because respiratory pathogens show varying resistance patterns warranting susceptibility testing,[34] culture still is necessary for expansive susceptibility testing, although some susceptibility information might be gathered through sequencing.[18]

Notably, we found that mNGS did not have any impact on 94 (59.1%) cases, mainly because mNGS did not detect any additional pathogens in the majority of these cases. This may be explained in part by the major limitation of this study, in which the standard methods did not include testing for RNA viruses other than influenza A/B, parainfluenza viruses 1 to 4, and respiratory syncytial virus, and the mNGS test was limited to sequence only DNA, not RNA. Because rhinovirus was shown to be one of the top causes of community-acquired pneumonia, it is very likely that many negative cases in this study had rhinovirus infections.[3] Other RNA viruses, such as human metapneumovirus and seasonal coronaviruses, also can cause pneumonia, although less frequently.[3] Sequencing both DNA and RNA in BAL will provide more complete respiratory pathogen detection and has been investigated in lower-respiratory-tract infections.[15] The second limitation was that the software used in this study assumed that any normal flora species with abundance (sequence reads) lower than a set threshold was deemed a contaminant and filtered out. This is an imperfect way to report results, given the complexity of lower-respiratory-tract infections and the ambiguity of the role of normal flora species, which may cause true infections in certain settings, even with low abundance.

Another limitation of this study was that the overall level of microbiology laboratory service in participating hospital systems may be relatively lower compared with more advanced laboratories. This is reflected by a significantly smaller test menu for BAL, as shown by the limited tests included in the standard methods in this study (Supplemental Figure S3). For example, the broad-spectrum respiratory pathogen PCR panel such as the Biofire FilmArray assay is not widely available in China, limiting the detection of many RNA viruses that may be involved in causing pneumonia in most hospitals. Thus, this study represents a specific comparison of mNGS with currently used methodologies in this particular region of China and may not be universally applicable, especially when compared with other advanced diagnostic technologies and differing standard methodologies used in other countries. However, this could provide a unique opportunity for mNGS to be used more widely in China owing to the tremendous clinical needs and the technological gap in molecular diagnostics. In resource-limited health care settings, a single wide-spectrum molecular assay, such as mNGS, may provide higher yield and improve clinical outcomes. Notably, the cost of an mNGS test in China has decreased to ¥2000 to 3000 (US ~$300 to $500), and is expected to decrease even more as sequencing cost continues to decrease. Although mNGS is becoming more affordable, it is important to note that the cost of mNGS does vary by country and may not be reimbursable.

In summary, we present the first large-scale prospective multicenter clinical study to fully evaluate the clinical utility of mNGS for the diagnosis of pneumonia and showed that mNGS of BAL provides significant value for improving the diagnosis of pneumonia and contributing to better patient care.

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Author Contributions

H.Z., P.M.K.L., D.Z., S.Y., and Y.Yu contributed to the design, data collection and analysis, and writing of the manuscript; Q.M., Y.Ya., X.W., J.W., X. Z., Y.L., G.W., M.F., L.W., J.C., C.Z., and J.Z. contributed to the data collection, data analysis, diagnosis, and treatment of patients; H.Z., X.H., and S.Y. contributed as data managers,

building the database, and in data analysis; and H.Z., S.Y., and Y.Yu contributed to the interpretation of data results and revision of the manuscript.

Supplemental Data

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Supplemental Figure S1 Distribution of total sequence reads and nonhuman sequence percentage of the 159 bronchoalveolar lavage (BAL) samples. AFB, acid-fast bacilli; CMV, cytomegalovirus; DFA, direct fluorescence antibody stain; mNGS, metagenomics next-generation sequencing; MTB, *Mycobacterium tuberculosis*; PJP, *Pneumocystis jirovecii*.

Supplemental Figure S2 Normal flora detected in the 159 bronchoalveolar lavage samples.

Supplemental Figure S3 Microbiological, serologic, and molecular methods used in this study.