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MAJOR ARTICLE

Applicability of Bronchoalveolar Lavage Fluid and Plasma Metagenomic Next-Generation Sequencing Assays in the Diagnosis of Pneumonia

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Background. Metagenomic next-generation sequencing (mNGS) provides innovative solutions for predicting complex infections. A comprehensive understanding of its strengths and limitations in real-world clinical settings is necessary to ensure that it is not overused or misinterpreted.

Methods. Two hundred nine cases with suspected pneumonia were recruited to compare the capabilities of 2 available mNGS assays (bronchoalveolar lavage fluid [BALF] mNGS and plasma mNGS) to identify pneumonia-associated DNA/RNA pathogens and predict antibiotic resistance.

Results. Compared to clinical diagnosis, BALF mNGS demonstrated a high positive percent agreement (95.3%) but a low negative percent agreement (63.1%). Plasma mNGS revealed a low proportion of true negatives (30%) in predicting pulmonary infection. BALF mNGS independently diagnosed 65.6% (61/93) of coinfections and had a remarkable advantage in detecting caustic, rare, or atypical pathogens. Pathogens susceptible to invasive infection or bloodstream transmission, such as *Aspergillus* spp, *Rhizopus* spp, *Chlamydia psittaci*, and human herpesviruses, are prone to be detected by plasma mNGS. BALF mNGS tests provided a positive impact on the diagnosis and treatment of 128 (61.2%) patients. Plasma mNGS, on the other hand, turned out to be more suitable for diagnosing patients who received mechanical ventilation, developed severe pneumonia, or developed sepsis (all $P < .01$). BALF mNGS was able to identify resistance genes that matched the phenotypic resistance of 69.4% (25/36) of multidrug-resistant pathogens.

Conclusions. Our data reveal new insights into the advantages and disadvantages of 2 different sequencing modalities in pathogen identification and antibiotic resistance prediction for patients with suspected pneumonia.

Keywords. antibiotic resistance gene; metagenomic next-generation sequencing; mNGS; pneumonia.

Community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP) are the leading causes of morbidity and mortality worldwide. Due to the limitations of conventional pathogen detection methods and the complex atypical clinical manifestations, there are still a large number of undiagnosed pneumonia cases in hospitalization and community settings. To make matters worse, antimicrobial resistance is responsible for increasing rates of treatment failure in patients with pneumonia, especially HAP [1]. Hence, accurate and timely identification of pathogens and prediction of their resistance to antimicrobials is of utmost importance to save pneumonia patients and reduce the socioeconomic burden.

In recent years, metagenomic next-generation sequencing (mNGS) has become a promising method for identifying complex infections due to its advantages in identifying a broad spectrum of pathogens, predicting antibiotic resistance, and providing test results within 24 hours [2, 3]. Previous proof-of-concept studies have shown that mNGS on lower respiratory tract samples such as bronchoalveolar lavage fluid (BALF) increased the positive rate of samples (>60%), significantly higher than that of conventional microbiological detection methods (30%–50%) [4, 5]. Of interest, another assay modality, namely plasma mNGS testing (ie, a liquid biopsy modality by sequencing cell-free nucleic acids from plasma), has also been shown to be potentially useful in predicting pneumonia-associated pathogens [6–8].

However, in real-life scenarios involving patients with suspected pneumonia, urgent answers are needed to address the following essential clinical inquiries: (1) For which microbial taxa does mNGS testing offer more utility? (2) Which patient subgroups derive the most benefit from mNGS? (3) How many patients experience a positive impact from mNGS in terms of diagnosis and treatment? One of our recent studies

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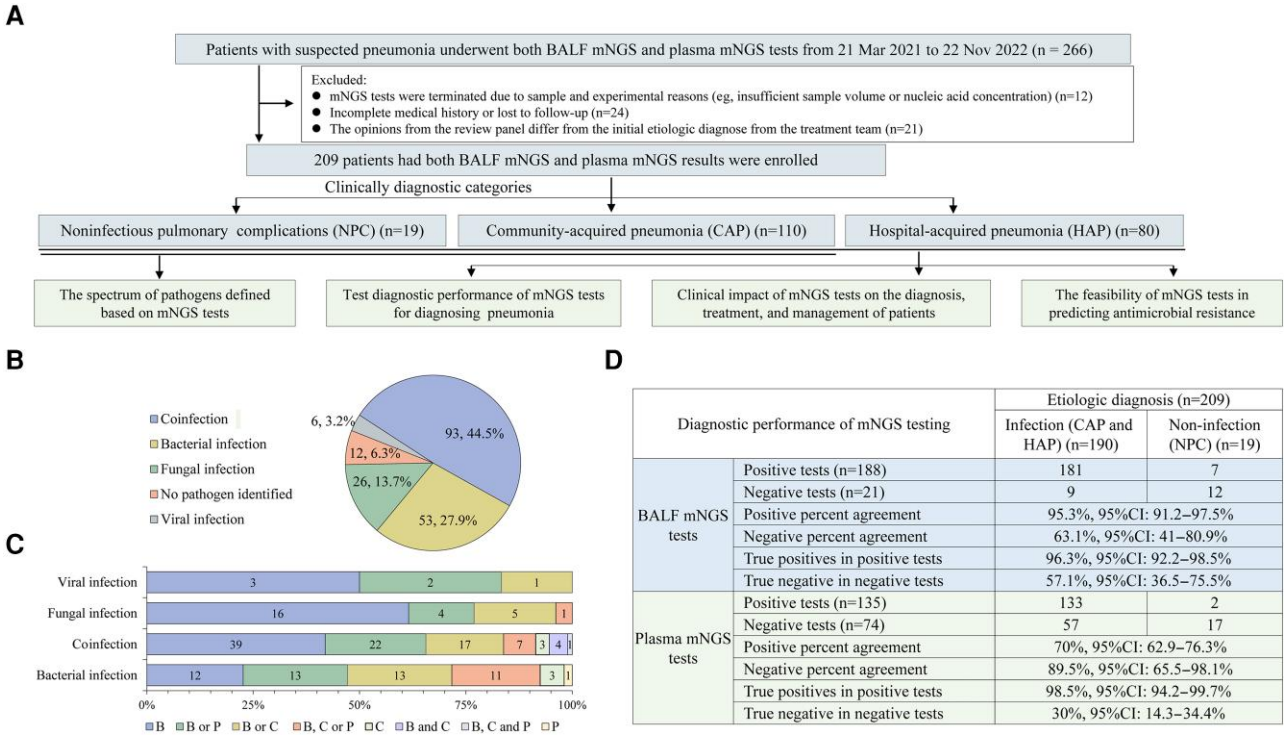

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Diagnostic performance of mNGS testing		Etiologic diagnosis (n=209)	
		Infection (CAP and HAP) (n=190)	Non-infection (NPC) (n=19)
BALF mNGS tests	Positive tests (n=188)	181	7
	Negative tests (n=21)	9	12
	Positive percent agreement	95.3%, 95%CI: 91.2–97.5%	
	Negative percent agreement	63.1%, 95%CI: 41–80.9%	
	True positives in positive tests	96.3%, 95%CI: 92.2–98.5%	
	True negative in negative tests	57.1%, 95%CI: 36.5–75.5%	
Plasma mNGS tests	Positive tests (n=135)	133	2
	Negative tests (n=74)	57	17
	Positive percent agreement	70%, 95%CI: 62.9–76.3%	
	Negative percent agreement	89.5%, 95%CI: 65.5–98.1%	
	True positives in positive tests	98.5%, 95%CI: 94.2–99.7%	
	True negative in negative tests	30%, 95%CI: 14.3–34.4%	

Figure 1. Study design and diagnostic performance of metagenomic next-generation sequencing (mNGS) tests. *A*, Enrolled patients and study design. *B*, Clinical diagnoses of enrolled patients. *C*, Methods for identifying pathogen infections in patients infected with different pathogens (B indicates bronchoalveolar lavage fluid mNGS; P, plasma mNGS; C, conventional tests). *D*, Diagnostic performance of mNGS testing.
Abbreviations: BALF, bronchoalveolar lavage fluid; CAP, community-acquired pneumonia; CI, confidence interval; HAP, hospital-acquired pneumonia; mNGS, metagenomic next-generation sequencing; NPC, noninfectious pulmonary complication.

evaluating the clinical utility of plasma mNGS testing in real clinical practice showed that only 57% of tests were considered helpful for patient care, which is higher than the results of previous studies [9–12], but still around 40% of the mNGS tests were of unclear or no impact for patient diagnosis and management. Therefore, further prospective observational studies performed in real-world practice are necessary to determine the appropriate testing patients and optimal timing of adoption to improve the clinical utility of mNGS.

In this study, we conducted a prospective observational study to evaluate the clinical impact of mNGS on BALF and plasma samples in the differential diagnosis of patients with clinically suspected pneumonia, providing first-line clinical evidence for the proper use of this complex pathogen identification tool in DNA and RNA pathogen detection and antibiotic resistance prediction.

METHODS

Study Population and Ethical Considerations

Between 21 March 2021 and 22 November 2022, 209 patients with suspected pneumonia admitted to the First Affiliated Hospital, Zhejiang University School of Medicine, were enrolled in this study (Figure 1*A*). All individuals underwent both BALF and plasma mNGS assays simultaneously to identify potential DNA and RNA pathogens. Similar to our recent studies [2, 13, 14], the etiologic diagnosis (ie, CAP, HAP, and noninfectious pulmonary complications [NPCs]) of each patient was first determined by the clinical treatment team according to laboratory data such as microbiological, biochemical, immunological, hematological, and oncological tests; radiology results; clinical manifestations; epidemiology history; treatment; disease prognosis; and several published guidelines [15, 16]. To minimize diagnostic bias due to differences in the competence of different clinical treatment teams, the initial etiologic diagnoses of all cases were further reviewed by a panel consisting of 3 authors (Y. C., who specializes in infectious diseases, and Q. Y. and D. H., who specialize in clinical microbiology). If their opinions differed from the initial etiologic diagnosis of the treatment team, the corresponding case was excluded from the study. The definition of immunosuppression was established through a published consensus [17]. This study was approved by the Institutional Review Board of the First Affiliated Hospital, Zhejiang University School of Medicine (IIT20220714A).

Conventional Microbiological Tests

Clinicians prescribed conventional microbiological tests based on each patient's specific needs. These tests include bacterial

and fungal cultures; smears; stains such as acid-fast stain, fluorescent stain, and India ink stain; serological tests for pathogens such as *Legionella* spp, *Cryptococcus* spp, *Candida* spp, and *Aspergillus* spp; and polymerase chain reaction (PCR) for human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and respiratory viruses such as respiratory syncytial virus, rhinovirus, influenza virus, and parainfluenza virus.

Microbial Identification and Resistance Gene Detection by mNGS Tests

BALF and plasma mNGS tests were performed in our clinical laboratory to detect both DNA and RNA pathogens. Our recent studies provide a comprehensive description of the analytical and clinical validation of tests, as well as the experimental procedures (eg, sample processing, nucleic acid extraction, library construction, high-throughput sequencing, and bioinformatics analysis), reagents, controls, sequencers, software, and algorithms involved in each step [2, 13, 14, 18, 19]. The detected pathogens and the number of sequencing reads (stringent mapped read number [SMRN], referring to the number of reads per pathogen at the sequencing depth of 20 million reads) are sent to the clinical treating team from the clinical laboratory [2].

For antibiotic resistance gene (ARG) prediction, the sequencing data after removing human sequences will be directly aligned to the ARG reference database (CARD: https://card.mcmaster.ca/) using BLASTN with parameters (-megablast, -evalue 1e-5) to determine the presence of ARGs associated with clinically common multidrug-resistant (MDR) bacteria, such as extended-spectrum β-lactamase (ESBL)–encoding genes (*bla*TEM-1, *bla*SHV-1, and *bla*CTX-M) and carbapenemase-encoding genes (*bla*KPC, *bla*NDM, *bla*VIM, *bla*OXA-23, *bla*OXA-51, and *bla*OXA-48) in gram-negative Enterobacteriaceae (eg, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*), the methicillin resistance gene (*mecA*) in *Staphylococcus aureus*, and vancomycin-resistant genes (*vanA*, *vanB*, and *vanC*) in enterococci isolates. Theses clinically relevant genes were predicted and filtered out based on the pathogen identified by mNGS. Clinically relevant gene alignments with <90% coverage were removed and only resistance genes with >1 gene alignment were reported to remove any possible bioinformatics errors.

Statistical Analysis

Demographic data were summarized using descriptive statistics. We used the methods described in our recently published study to evaluate the diagnostic performance of mNGS testing and the clinical impact of plasma mNGS testing [2]. The generation of figures and tables relies on GraphPad version 8.0.1 software (GraphPad Software, La Jolla, California) and Microsoft Office Excel 2021. Statistical tests were performed using SPSS20.0 software (IBM, Armonk, New York). *P* values <.05 were considered significant, and all tests were 2-tailed.

RESULTS

Clinical Characteristics

Of the enrolled patients, 73.2% (153/209) had poor underlying conditions such as hematologic disorders (29.9%), solid organ transplantation (18.4%), and cancer (14.3%) (Table 1). More than half of the patients were immunocompromised (65.1%), required intensive care unit (ICU) admission (78.5%), received invasive mechanical ventilation (80.4%), or developed severe pneumonia (66%). Sepsis occurred in 46% of patients, and 90.9% (190/209) of patients were diagnosed with CAP (n = 110) or HAP (n = 80) (Figure 1*A*). Coinfection, bacterial, and fungal infections occurred in 44.5% (93/190), 27.9% (53/190), and 13.7% (26/190) of cases, respectively. Viral infections were confirmed in only 6 patients (Figure 1*B*). The remaining 19 patients were classified as NPCs (ie, 5 hypersensitivity pneumonitis, 5 acute cardiogenic pulmonary edema, 4 organizing pneumonia, 2 drug-induced interstitial lung disease, 2 acute lung injury secondary to sepsis, and 1 leukemic pulmonary infiltrate) (Supplementary Material).

Diagnostic Performance of mNGS for Predicting Pneumonia

BALF mNGS alone identified all the causative pathogens for 36.8% (n = 70) of the 190 patients with CAP or HAP (Figure 1*B*), including 65.6% (61/93) of coinfections, 61.5% (16/26) of fungal infections, 50% (3/6) of viral infections, and 47.2% (25/53) of bacterial infections. The conventional microbiologic tests alone identified the pneumonia-causing pathogens in 6 patients (coinfection: P26, P68, and P117; bacterial infection: P8, P116, and P179) (Figure 1*B*, Supplementary Material). Only 1 patient with pulmonary tuberculosis (P27) had the pathogen (*Mycobacterium tuberculosis*) identified using plasma mNGS alone. The pathogens in 5 patients (3 HAP: P2, P23, and P25; 2 CAP: P37 and P155) were confirmed by a combination of conventional and BALF mNGS tests (see Supplementary Material). The remaining cases involve 60 (31.6%) infections with pathogens identified by BALF or plasma mNGS tests and 55 (28.9%) infections with pathogens detected by conventional, BALF mNGS, or plasma mNGS tests.

BALF mNGS, using a clinical diagnosis as a reference standard, revealed a high positive percent agreement (95.3%) and the proportion of true positives (96.3%) out of positive mNGS tests in predicting pulmonary infection, but its negative percent agreement (63.1%) and proportion of true negatives (57.1%) were relatively low. In comparison, plasma mNGS had a lower positive percent agreement (70%) and a lower proportion of true negatives (30%) (Figure 1*C*).

The Spectrum of Pneumonia-Causing Pathogens Identified With mNGS Tests

BALF mNGS tests identified a total of 368 pneumonia-causing pathogens (87 species) from 162 patients in this study, including 170 bacteria (46 species), 78 fungi (17 species), and 107

Table 1. Demographic and Clinical Characteristics of the Enrolled Patients

Characteristics	No. (%)
Patient demographics (n = 209)	
Age, y	
Median (IQR)	62 (51–71)
Range	1–90
Sex	
Female	74 (35.4)
Male	135 (64.6)
Primary medical condition	
Solid organ transplant	40 (19.1)
Lung transplant	15 (7.2)
Kidney transplant	11 (5.3)
Liver transplant	9 (4.3)
Heart transplant	5 (2.4)
Hematological diseases	42 (20.1)
Leukemia	26 (12.4)
Lymphoma	10 (4.8)
Myelodysplastic syndrome	4 (1.9)
Multiple myeloma	2 (1.0)
Cancer	25 (12.0)
Cardiovascular diseases	11 (5.3)
COPD	9 (4.3)
Diabetes	17 (8.1)
Autoimmune disease	6 (2.9)
HIV	3 (1.4)
No underlying medical condition	56 (28.2)
ICU admission	
Yes	164 (78.5)
No	45 (21.5)
Immunocompromised	
Yes	136 (65.1)
No	73 (34.9)
Mechanical ventilation	
Yes	168 (80.4)
No	41 (19.6)
Severe pneumonia	
Yes	138 (66.0)
No	71 (44.0)
Sepsis	
Yes	96 (46.0)
No	113 (54.0)
Final diagnosis	
Community-acquired pneumonia	110 (52.6)
Hospital-acquired pneumonia	80 (38.3)
Noninfectious pulmonary complications	19 (9.1)
Outcome	
Recovery	118 (56.5)
Death	91 (43.5)

Data are presented as No. (%) unless otherwise indicated.

Abbreviations: COPD, chronic obstructive pulmonary disease; HIV, human immunodeficiency virus; ICU, intensive care unit; IQR, interquartile range.

viruses (21 species). Conventional microbiological methods (culture and PCR) confirmed the presence of 51.8% (88/170) of bacteria, 30.8% (24/78) of fungi, and 22.4% (24/107) of viruses (Figure 2), including more than 50% of *K pneumoniae*, *P aeruginosa*, *A baumannii*, *Stenotrophomonas maltophilia*, *E coli*, and *Candida albicans* that were also identified using cultural methods. While the clinical caustic pathogens such as *Chlamydia psittaci*, *Legionella pneumophila*, *Ureaplasma urealyticum*, anaerobes, *Pneumocystis jirovecii*, *Cryptococcus neoformans*, *Rhizopus* spp, and various viruses were only identified by mNGS tests due to their inherent difficult-to-cultivate properties or lack of targeted detection techniques in the clinical laboratory.

CAP-associated pathogens (74 species) outnumbered HAP-associated pathogens (47 species). *Chlamydia psittaci* (n = 5), *M tuberculosis* (n = 4), *Nocardia farcinica* (n = 3), *Vibrio vulnificus* (n = 1), *Talaromyces marneffei* (n = 1), *Orientia tsutsugamushi* (n = 1), and *Rhizopus* spp (*R delemar*, *R microspores*, and *R tardus*) were only found in CAPs.

The paired plasma mNGS tests showed 160 pathogens (49 species) of the 355 pneumonia-associated pathogens in 94 patients, with 51% (15/29) of *K pneumoniae*, 78.1% (57/73) of human herpesviruses (HHV-1, HHV-2, varicella zoster virus, EBV, HCMV, and HHV-6B), 60% (12/20) of *Aspergillus* spp (*A fumigatus* and *A flavus*), all *Rhizopus* spp (*R delemar*, *R microspores*, and *R tardus*) (n = 4), and *C psittaci* (n = 5), as shown in Figure 2.

Clinical Impact of BALF mNGS Tests on Patient Management

The BALF mNGS tests showed a positive impact on the diagnosis and treatment of 128 (61.2%) patients (Table 2), including helping patients establish a new diagnosis and initiating targeted anti-infection therapy (n = 76), upgrading antimicrobial regimens (n = 31), withdrawing unnecessary antibiotic use (n = 10), and ruling out infection (n = 11). However, nearly 40% (n = 81) of patients did not change any treatment or management based on the mNGS results because (1) empirical antibiotic regimens before mNGS testing covered the identified pathogens (ie, no need to adjust the antibiotic regimen) (n = 34); (2) conventional tests identified the pathogens earlier and initiated appropriate therapy (n = 26); (3) mNGS results could not explain the clinical presentation of patients (ie, false-negative or false-positive results) (n = 17); and (4) lack of drugs available for treatment (eg, viral infections) (n = 4) (Table 2, Supplementary Material).

Clinical Impact of Plasma mNGS Tests in Predicting Pneumonia

Plasma mNGS identified pneumonia-associated pathogens in 45% (94/209) of the enrolled cases; it accurately predicted all pathogens causing pulmonary infections in 60 (28.7%) patients, detected not only pathogens of pulmonary origin but also those causing intestinal infections in 2 patients, and identified only some but not all of the pathogens responsible for pulmonary infections in the other 32 patients (Figure 3*A*). Further analysis demonstrated that among these 94 patients, those who received mechanical ventilation (n = 168, $P < .01$), developed severe pneumonia (n = 96, $P < .01$), or developed sepsis (n = 138, $P < .01$) had a higher positive rate of plasma mNGS than that of other patients who did not have these underlying health

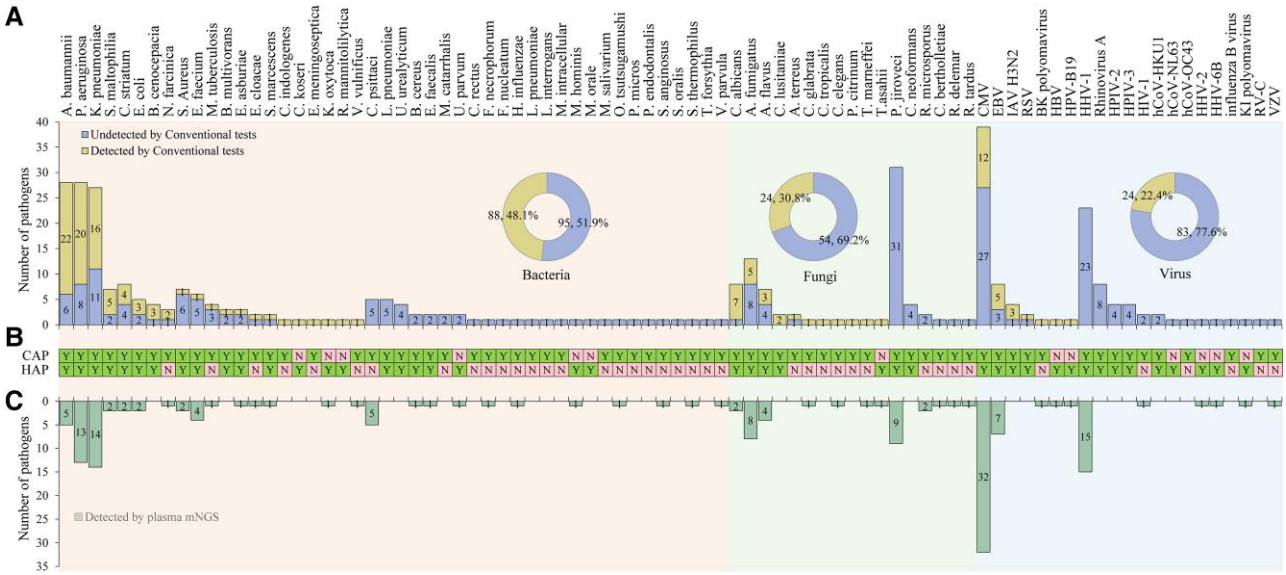


Figure 2. The spectrum of pneumonia-causing pathogens identified through bronchoalveolar lavage fluid (BALF) metagenomic next-generation sequencing (mNGS) tests. *A*, The column chart displays all the pathogens identified by BALF mNGS. The 3 pie charts depict the total proportion of bacteria, fungi, and viruses identified by BALF mNGS that can also be detected by conventional tests. *B*, The box plots display the distribution of each pathogen in the upper column chart in patients with community-acquired pneumonia and hospital-acquired pneumonia. *C*, The column chart displays the detection of each pathogen using plasma mNGS in the upper bar graph. The lower column chart displays which pathogens detected by BALF mNGS were also detected by plasma mNGS.

Abbreviations: CAP, community-acquired pneumonia; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HAP, hospital-acquired pneumonia; HBV, hepatitis B virus; hCoV, human coronavirus; HHV, human herpesvirus; HIV, human immunodeficiency virus; HPIV, human parainfluenza virus; HPV, human papillomavirus; IAV, influenza A virus; mNGS, metagenomic next-generation sequencing; N, not detected; RSV, respiratory syncytial virus; RV-C, rhinovirus C; VZV, varicella zoster virus; Y, detected.

Table 2. Clinical Impact of Bronchoalveolar Lavage Fluid Metagenomic Next-Generation Sequencing Tests

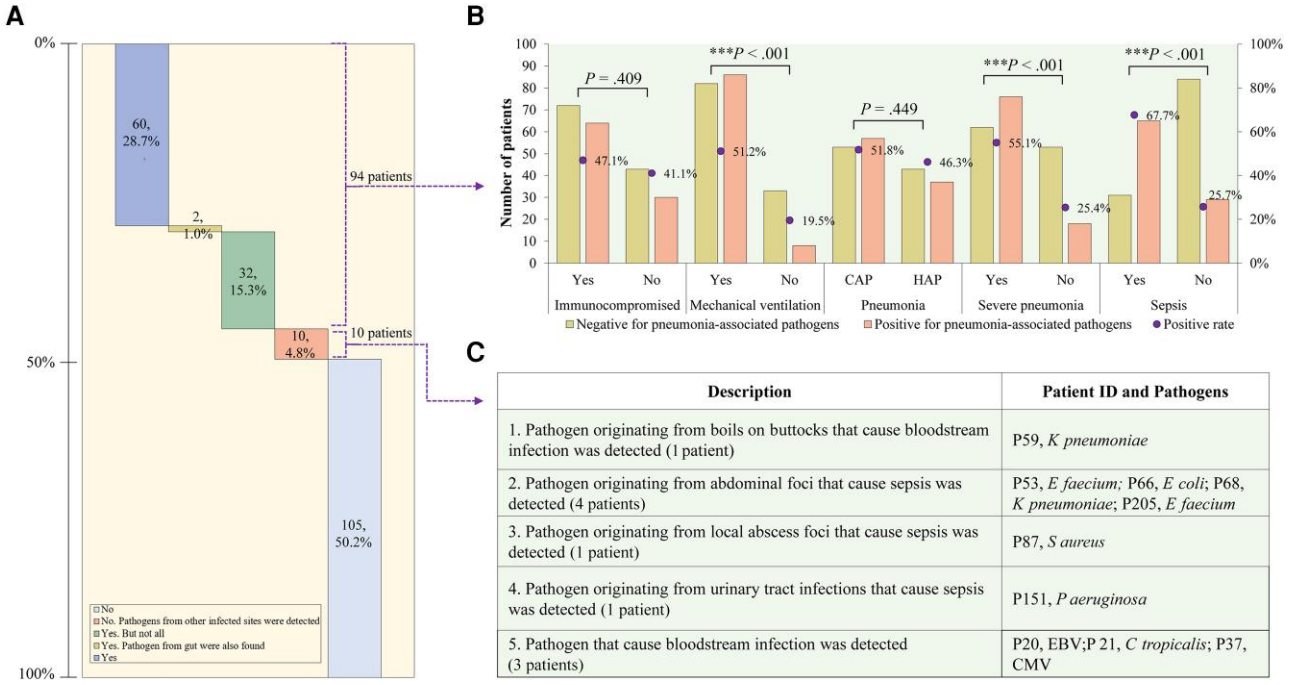
Category of Clinical Impact	No. (%)
Positive impact	128 (61.2)
Enabled new diagnosis of infection and initiation of targeted therapy	76 (36.4)
Enabled new diagnosis of infection and escalation of therapy	31 (14.8)
Enabled new diagnosis of infection and de-escalation of therapy	10 (4.8)
Enabled ruling out of infection and initiation of noninfectious therapy	11 (5.3)
No impact	81 (38.8)
Redundant information; antibiotics and clinical plan were not changed	77 (36.8)
Enabled new diagnosis of infection but targeted treatment has not been initiated (due to lack of therapeutic drugs or severe illness)	4 (1.9)

conditions (Figure 3*B*). It is noteworthy that in 10 patients, plasma mNGS did not detect any pathogen associated with pulmonary infection, but instead found pathogens from other sites of infection (Figure 3*C*), which is another benefit of plasma mNGS testing.

Antibiotic Resistance Gene Detection by mNGS Tests

We compared mNGS-identified genotypic resistance with phenotypic culture results for the most prevalent 4 pathogens (61 strains in total, including 22 *A baumannii*, 20 *P aeruginosa*, 16 *K pneumoniae*, and 3 *E coli*) detected by both BALF mNGS and microbial culture (Figure 4*A*). A total of 36 MDR pathogens were screened using culture-based antimicrobial susceptibility testing, of which 25 (69.4%) strains matched the genotypes detected by BALF mNGS. Specifically, mNGS-detected resistant genotypes were able to predict 84.2% (16/19) of carbapenem-resistant *A baumannii*, while only predicting 40% (2/5) of carbapenem-resistant *P aeruginosa* and 60% (6/10) of carbapenem-resistant *K pneumoniae* (CRKP) and ESBL *K pneumoniae*. We found that the sequenced SMRN values of all MDR strains with no ARGs detected by BALF mNGS were below the median SMRN values, which suggests that sequencing depth is an important factor affecting detection sensitivity (Figure 4*B*–*D*). Overall, the agreement between the genotypes detected by BALF mNGS and the reported phenotypic resistances by the microbial culture of 61 strains can reach 80.3% (49/61) (Figure 4*A*).

The plasma mNGS identified ARGs (KPC gene and NDM gene) associated with the causing pathogen (*K pneumoniae*, SMRN value = 5992) in only 1 leukemia patient (P90) admitted to the ICU. The *K pneumoniae* strain was identified through microbiological culture as a CRKP strain that is resistant to the antibiotic ceftazidime-avibactam. Given that the KPC gene is the main reason for *K pneumoniae* resistance to carbapenem, and



Description	Patient ID and Pathogens
1. Pathogen originating from boils on buttocks that cause bloodstream infection was detected (1 patient)	P59, *K pneumoniae*
2. Pathogen originating from abdominal foci that cause sepsis was detected (4 patients)	P53, *E faecium;* P66, *E coli*; P68, *K pneumoniae*; P205, *E faecium*
3. Pathogen originating from local abscess foci that cause sepsis was detected (1 patient)	P87, *S aureus*
4. Pathogen originating from urinary tract infections that cause sepsis was detected (1 patient)	P151, *P aeruginosa*
5. Pathogen that cause bloodstream infection was detected (3 patients)	P20, EBV;P 21, *C tropicalis*; P37, CMV

Figure 3. Summary of the clinical impact of plasma metagenomic next-generation sequencing (mNGS) in predicting pneumonia. *A*, Performance of plasma mNGS for identifying pneumonia-associated pathogens. *B*, Among 94 patients with at least 1 pneumonia-associated pathogen detected by plasma mNGS, those who received mechanical ventilation or developed severe pneumonia or sepsis had a higher positive rate of plasma mNGS than that of other patients who did not have these underlying health conditions. *C*, In 10 patients, although plasma mNGS did not detect any pathogen associated with pulmonary infection, pathogens from other sites of infection were found. ***indicates significant differences between comparison groups. Abbreviations: CAP, community-acquired pneumonia; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HAP, hospital-acquired pneumonia.

A

Reported resistance phenotypes by microbial culture		Number of samples	Antibiotic resistance genes (ARGs) identified by BALF mNGS testing (Number of samples)	Agreement between ARGs and reported resistance phenotypes for MDR isolates	Agreement between ARGs and reported resistance phenotype for all isolates
22 *A baumannii* identified by mNGS					
MDR	CRAB	16	blaOXA-23 (1); blaOXA-51/blaOXA-23/blaTEM (7); blaOXA-51/blaOXA-23 (8)	16/19= 84.2%	18/22=81.8%
		3	Undetected (3)		
Other	Drug-susceptible *A baumannii* *	1	blaOXA-51 (1)	/	
		2	Undetected (2)	/	
20 *P aeruginosa* identified by mNGS					
MDR	CRPA	2	KPC/blaSHV (1); KPC (1)	2/5= 40%	17/20=85%
		3	Undetected (3)		
Other	Drug-susceptible *P aeruginosa* *	15	Undetected (15)	/	
16 *K Pneumoniae* identified by mNGS					
MDR	CRKP	4	KPC/blaSHV/blaCTX-M/blaTEM (3); blaNDM/blaSHV/blaCTX-M/blaTEM (1)	6/10= 60%	12/16= 75%
		4	Undetected (4)		
	ESBL-KP	2	blaCTX-M (1); blaSHV/blaCTX-M/blaTEM (1)		
Other	Drug-susceptible *K pneumoniae* *	6	Undetected (6)	/	
3 *E coli* identified by mNGS					
MDR	ESBLs-ECO	1	blaCTX-M (1)	1/2= 50%	2/3=66.7%
		1	Undetected (1)		
Other	Drug-susceptible *E coli* *	1	Undetected (1)	/	
Total	/	62	/	25/36= 69.4%	49/61=80.3%

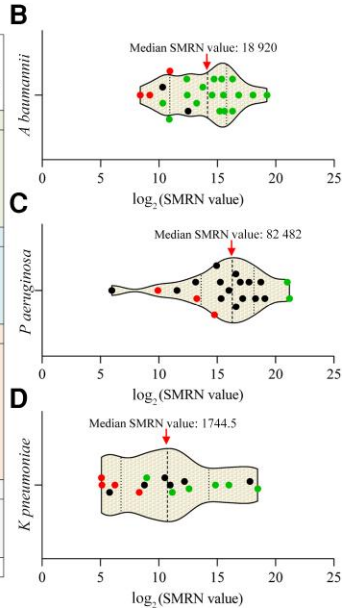


Figure 4. Antibiotic resistance gene detection using metagenomic next-generation sequencing (mNGS) tests. *A*, The comparison of mNGS-identified genotypic resistance with phenotypic culture results for the 4 most prevalent pathogens. * indicates no detection of multidrug resistance. *B–D*, Distribution of the stringent mapped read number of *Acinetobacter baumannii* (*A*), *Pseudomonas aeruginosa* (*B*), and *Klebsiella pneumoniae* (*C*). Red dots are multidrug-resistant strains for which no resistance genes were detected; green dots are multidrug-resistant bacteria for which resistance genes were detected; black dots are phenotypically susceptible strains.
Abbreviations: ARG, antibiotic resistance gene; BALF, bronchoalveolar lavage fluid; CRAB, carbapenem-resistant *Acinetobacter baumannii*; CRKP, carbapenem-resistant *Klebsiella pneumoniae*; CRPA, carbapenem-resistant *Pseudomonas aeruginosa*; ESBL-ECO, extended-spectrum β-lactamase *Escherichia coli*; ESBL-KP, extended-spectrum β-lactamase *Klebsiella pneumoniae*; MDR, multidrug-resistant; mNGS, metagenomic next-generation sequencing; SMRN, stringent mapped read number.

the NDM gene encoding New Delhi metallo-β-lactamase is one of the mechanisms of ceftazidime-avibactam resistance, we determined that the genotype of plasma mNGS and drug resistance phenotype are consistent.

DISCUSSION

Metagenomic next-generation sequencing has greatly improved the diagnosis of clinical complex infections, and the way to ensure it is not overused or misinterpreted in disease diagnosis is to fully understand its advantages and limitations in real clinical settings. This real-world clinical study compared the capabilities and challenges of BALF and plasma mNGS in pathogen identification and drug resistance prediction for >200 patients with suspected pneumonia. The results showed that BALF mNGS has high sensitivity (95.3%) but inferior specificity (63.1%) for the diagnosis of pneumonia, similar to the results reported by our and other teams [2, 20–23]. The underlying reason for the low specificity is the false-positive issue with this complex assay. On the one hand, mNGS is at risk of false positives due to cross-contamination between samples; incidental contaminants from the laboratory environment, reagents, and consumables; and incorrect classification or typing of species due to inaccuracies in the bioinformatics analysis pipeline, which has been thoroughly discussed in other studies [24–26]. On the other hand, in clinical settings, the results of an mNGS assay are often polymicrobial, especially with the diversity of opportunistic pathogenic microorganisms in the respiratory tract, making it challenging to determine if they are truly clinically relevant (colonized or infected). Despite adhering to standard operating procedures and quality control monitoring in this study, 16% (70/368) of microorganisms detected by BALF mNGS were eventually not recognized as the responsible pathogen (ie, irrelevant pathogen) due to inconsistencies with the clinical presentation of the patients (Supplementary Material). Therefore, for a more accurate assessment of the clinical significance of microbes detected by mNGS, it is crucial to emphasize the integration of microbial biological characteristics with the patients' clinical presentations and to conduct necessary infectious disease consultations [10].

Given its ability to detect multiple microorganisms, mNGS has an inherent advantage in predicting coinfections. In the current study, BALF mNGS was able to independently resolve more than 60% (39 cases) of coinfections, with the majority of these patients (71.4% [55/77 cases]) being immunocompromised due to solid organ transplantation, human immunodeficiency virus, or hematologic disorders. mNGS tests are particularly useful when there are no targeted techniques for detecting certain pathogens in the laboratory. For instance, due to the lack of rapid tests (eg, PCR) for *P jirovecii* other than fluorescent staining methods within our clinical laboratory, all 31 patients with interstitial pneumonitis due to *P jirovecii* in the present study were detected by mNGS. Pathogens such as *C psittaci*, *L pneumophila*, *U urealyticum*, *Ureaplasma parvum*, anaerobes, *C neoformans*, Mucorales, and various viruses were mainly detected by BALF mNGS alone, highlighting the advantages of mNGS in identifying atypical pathogens [27]. For plasma mNGS, we demonstrated that, when predicting pathogens in lung infections, it was effective in identifying pathogens that predispose to invasive infections such as *Aspergillus* spp (*A fumigatus* and *A flavus*) and *Rhizopus* spp (*R delemar*, *R microspores*, and *R tardus*), or pathogens whose infection cycle involves bloodstream dissemination such as *C psittaci* and human herpesviruses (eg, HHV-1, EBV, and HCMV).

In the present study, the result that 61.2% of BALF mNGS results led to positive clinical impact is similar to that reported by Yang et al, where 58% (47/81) of BALF mNGS results were considered clinically actionable [28], but higher than that reported by most other studies on evaluating BALF or plasma mNGS (7.3%–47%) [9–12, 29]. This may be due to the fact that clinicians in our hospitals typically use this complex and expensive assay in clinical scenarios where (1) there is an urgent need to clarify or exclude infections in critically ill patients, (2) when there is a lack of laboratory diagnostics for detecting potential pathogens, or (3) when it is difficult for available tests to give results within 1 day [2]. In such clinical situations, mNGS test results obtained within 24 hours often serve as crucial evidence for early diagnosis and treatment.

Nonetheless, nearly 40% (n = 81) of the BALF mNGS results have not significantly impacted the management of the patients, apart from possibly increasing confidence of the physicians to continue with the original treatment regimen. Of these cases, 26 had already been detected by conventional methods before the mNGS assay and 34 were effective despite the absence of pathogens with empiric therapy. Further evaluation and recommendations are needed to determine the necessity of expensive mNGS testing in these cases. As for plasma mNGS, only about 50% of tests were able to detect some or all of the pathogens that cause pulmonary infections, and our results suggest that it is more suitable for use in patients suffering from severe pneumonia, sepsis, and device-assisted ventilation because of its higher detection rate.

Several studies have indicated that the prediction of pathogen resistance using mNGS may be possible [30, 31]. Charalampous et al demonstrated the ability of mNGS to accurately predict phenotypic β-lactam resistance in culturable organisms (Enterobacterales or *Acinetobacter* spp) from nearly 95% (19/20) of respiratory samples [32]. In the present study, BALF mNGS genotypes and reported phenotypic resistance were matched in 80.3% (49/61) of pathogens, demonstrating the clinical utility of mNGS for rapid prediction of the presence or absence of resistant genes carried by main clinical pathogens.

As illustrated in Figure 4*B*, the SMRN values of MDR pathogens that missed resistance genes were low, which suggests that the sequencing depth may be a significant factor affecting ARG detection sensitivity. Furthermore, prediction errors due to sequencing errors or plasmid-borne resistance can also affect the accuracy of the detection results, but these challenges are expected to be overcome through technological advancements [33, 34]. Plasma mNGS measures fragmented nucleic acid fragments in a sample, making it more challenging to predict drug resistance genes [6, 11]. In this study, ARGs associated with phenotypic resistance of MDR bacteria were detected in only 1 patient, who was infected with a CRKP strain, highlighting the limited significance of plasma mNGS in predicting resistance at this stage.

Although this study is the first to comprehensively compare the clinical suitability of BALF and plasma mNGS in predicting pulmonary infections in a cohort of >200 patients, it is not without limitations. First, although a review panel was established to further evaluate the primary etiologic diagnosis based on comprehensive patient profiles by the treatment teams, subjective judgment bias may persist due to the complexity of the patients' clinical presentation. Second, this was a single-center observational study, and the findings should be confirmed in further studies with similar designs. Third, except for *A baumannii*, *P aeruginosa*, *K pneumoniae*, and *E coli*, other clinically important bacteria such as *S aureus* and *Enterococcus* were too few to compare the consistency of their mNGS genotypes and antimicrobial resistance phenotypes. This needs to be observed by more mNGS results in the future.

In summary, this study demonstrates that BALF mNGS is significantly different from plasma mNGS in its ability to predict pulmonary infections and resistance to MDR pathogens. When pulmonary infection–related samples, such as BALF, are not clinically available, plasma mNGS assays can be used to predict pulmonary infection, but relatively more reliable results are likely to only be obtained when used in patients with mechanical ventilation or who develop sepsis or severe pneumonia, or when infections with high-invasive-capacity pathogens are suspected. Although current mNGS assays have challenges such as low specificity due to false-positive issues, in any case, the advantages of this novel pathogenic molecular detection technology in the identification of clinically rare and atypical pathogens, as well as in the diagnosis of coinfections, are irreplaceable. In the era of rapid clinical translation of this technology, more promising studies demonstrating the suitability of mNGS tests in infectious diseases are needed to improve their clinical utility in real-world use.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. Study design: D. H. and Y. C. Sample detection: D. H., F. Y., and R. S. Data collection: D. H., R. S., D. Z., and F. Y. Data analysis: D. H., Q. Y., Y. C., and S. Z. Manuscript writing: D. H. All authors have read and approved the final version of the manuscript.

Data availability. Due to patient privacy and consent issues, we were unable to share the raw sequencing data used in this article to evaluate the mNGS results. Additionally, due to intellectual property concerns, the methods available to others to reproduce the results are limited. Taking into account these 2 limitations, the data used to draw the conclusions of this study have been included in the manuscript and the Supplementary Material.

Patient consent. This study only analyzes the test results of samples and does not publish the private information of the enrolled patients, so the informed consent of patients or their guardians was waived.

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