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MAJOR ARTICLE

Performance of mp-tNGS in Bronchoalveolar Lavage Fluid for the Diagnosis of Invasive Pulmonary Aspergillosis in Nonneutropenic Patients

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Background. Multiplex polymerase chain reaction (PCR)-based targeted next-generation sequencing (tNGS) is a promising tool for distinguishing lower respiratory tract infections in clinical practice, and its detectable pathogen spectrum can cover more than 95% of clinical cases, but there is limited information on systematic evaluation of the clinical use of multiplex PCR-based tNGS (mp-tNGS) in invasive pulmonary aspergillosis (IPA) cases. We aim to assess mp-tNGS in bronchoalveolar lavage fluid (BALF) for *Aspergillus* detection in patients with suspected IPA to provide a reliable basis for initiating antifungal therapy without microbiological or histopathological evidence.

Methods. We prospectively enrolled a cohort of consecutive patients with suspected IPA; all had undergone serum/BALF galactomannan antigen (GM), BALF mp-tNGS, and traditional tests (direct smear and culture of respiratory specimens). EORTC/MSG and FUDICU criteria or clinical compound diagnosis were used for IPA diagnosis.

Results. Thirty-two patients were diagnosed with IPA and 42 with non-IPA. Compared with the final diagnosis, the sensitivity of BALF mp-tNGS was 87.5%, while the sensitivities of traditional tests, serum GM, and BALF GM assay were 43.8%, 21.9%, and 62.5%, respectively. The specificity of BALF mp-tNGS was 90.5%, which was similar to traditional tests. The average turnaround time for *Aspergillus* detection by BALF mp-tNGS was 22.10 hours (SD 2.49 hours), which was significantly faster than traditional tests.

Conclusions. BALF mp-tNGS showed good performance in identification of *Aspergillus* in nonneutropenic IPA patients. Importantly, positive mp-tNGS in BALF can provide a basis for early antifungal therapy before microbiological evidence is available.

Keywords. multiplex PCR-based targeted next-generation sequencing (mp-tNGS); bronchoalveolar lavage fluid (BALF); invasive pulmonary aspergillosis (IPA); histopathology; galactomannan (GM).

Invasive pulmonary aspergillosis (IPA) is the leading cause of death from fungal infections, the most severe type of pulmonary aspergillosis, and has emerged as the second most common opportunistic invasive fungal infection after candidiasis [1]. IPA is generally prone to occur in immunocompromised populations [2, 3]. However, it has increasingly been recognized as an emerging disease in nonneutropenic individuals with predisposing conditions, such as corticosteroid treatment, chronic obstructive pulmonary disease, liver cirrhosis, and solid organ malignancy [4]. There is evidence that the incidence of IPA has been increasing in mildly to moderately immunodeficient populations in recent years [5]. Therefore, early diagnosis of IPA is essential for timely antifungal treatment and a good prognosis.

Traditional methods for detecting *Aspergillus* include bronchoalveolar lavage fluid (BALF)/sputum fungal culture and smear, and lung biopsy histopathology [6]. Histopathological examination through computed tomography (CT)-guided percutaneous lung biopsy or bronchoscopy biopsy usually provides a proven diagnosis for IPA when hyphae are identified by hematoxylin-eosin, periodic acid-Schiff, or Gomori methenamine silver staining [7, 8]. However, the risk of these invasive procedures is high or intolerable. In addition, histopathology requires a long turnaround time (TAT). Direct smear of BALF or sputum is a simple procedure without a long TAT but often yields low accuracy and specificity [9]. Positive cultures of *Aspergillus* from blood or sterile lung tissue samples can provide a proven diagnosis for IPA; positive cultures of *Aspergillus* in sputum/BALF combined with host factors and clinical images meet the criteria for probable IPA [10]. However, compared with histopathology, lung tissue culture has a longer TAT and lower sensitivity; although the specificity of microscopy and culture of *Aspergillus* in BALF can reach 97%, the sensitivity is only 30% to 50% [11].

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Serum and BALF galactomannan (GM) antigen are recognized as biomarkers of *Aspergillus* and are widely used in the diagnosis of aspergillosis. However, GM antigen detection is easily affected by antifungal treatment and leads to false negatives; in contrast, the use of β-lactam antibiotics may lead to false-positive results [12, 13]. Metagenomic next-generation sequencing (mNGS) can complement traditional diagnostic methods through high-throughput sequencing, detecting nucleic acids of pathogens directly from clinical samples, and then analyzing nucleic acid sequences through bioinformatics methods, which cover a wide range of pathogens, especially rare pathogens. Compared with traditional methods, mNGS achieves a higher detection rate of *Aspergillus* [14, 15]. However, it has the disadvantages of high costs, human gene interference, and DNA and RNA must be separately detected [16, 17]. mp-tNGS is a technology for high-throughput sequencing of specific gene sequences, which obtains the target nucleic acid fragments by multiplex polymerase chain reaction (PCR) amplification of the nucleic acids in the test sample with numerous of primers for specific gene sequences; finally, the obtained sequences are analyzed via bioinformatics [18, 19]. In our clinical practice, most of the pathogens that cause lower respiratory tract infection (LRTIs) are relatively common and tNGS can detect these relatively common pathogens simultaneously, with lower cost, fewer sequencing data, and higher efficiency, which can meet the needs of etiological diagnosis of most LRTIs. However, there are few studies on mp-tNGS for the detection of pathogenic microorganisms in LRTIs, especially *Aspergillus*.

METHODS

Diagnostic Criteria

The IPA diagnosis was divided into proven and probable diagnoses according to the consensus of the European Organization for the Research Treatment of Cancer/Mycoses Study Group (EORTC/MSG) [8] and Invasive Fungal Diseases in Adult Patients in Intensive Care Unit (FUNDICU) [20]; patients who lacked EORTC/MSG risk factors and were treated outside the ICU were diagnosed according to clinical compound diagnosis by a multidisciplinary panel of infection disease experts (M.W., T.R., and Y.T.).

Patients

From January 2022 to July 2024, a total of 101 patients with suspected IPA were enrolled in the study at Taihe Hospital. This study was approved by the Ethics Committee of Taihe Hospital, and performed in accordance with the principles of Good Clinical Practice following the Tri-Council guidelines. Informed written consent was obtained from each patient for participation in the study and publication of anonymized information.

According to the inclusion and exclusion criteria, 78 patients were ultimately enrolled in the study. The inclusion criteria were (1) age ≥18 years; (2) BALF samples were collected for mp-tNGS, GM detection, and microbial culture; (3) BALF mp-tNGS was performed before antifungal administration; (4) the numbers of reads per 100 000 sequencing reads of *Aspergillus* by mp-tNGS ≥ 100; (5) CT-guided percutaneous lung biopsy was performed in patients with negative sputum tests and no clear microbial evidence from bronchoscopy; (6) IPA diagnosis was in accordance with the criteria of EORTC/MSG and FUDICU or clinical compound diagnosis [8]; and (7) informed consent was obtained. The exclusion criteria were (1) undiagnosed; (2) possible IPA; and (3) unavailable data or missing large amounts of clinical data. The flowchart for the selection and diagnosis of the study population is shown in Figure 1.

BALF and Lung Biopsy Specimens

All patients underwent bronchoscopy with alveolar lavage. Bronchoscopic forceps biopsy was performed when necessary or lesions were visible. Percutaneous lung biopsy was performed when there was a negative sputum tests and no clear micro evidence from bronchoscopy, and specimens collected from the lesion sites were sent for pathology. These procedures were performed as in previous publications [21, 22]. Each BALF sample was divided into 3 parts for mp-tNGS analysis, GM assay, and conventional tests (direct smear, culture). Informed consent was obtained before bronchoscopy or percutaneous lung biopsy.

Serum and BALF GM Antigen Tests

The Platelia *Aspergillus* double sandwich enzyme-linked immunosorbent assay kit (Bio-Rad) was used for qualitative analysis of *Aspergillus* GM antigen in serum and BALF according to the manufacturer's instructions [23]. Optical density (ODI) in serum and BALF refers to the sample/standard value according to the manufacturer's protocol. In our study, we defined an ODI ≥ 1.0 in BALF and an ODI ≥ 0.6 in serum as the optimal cutoff values for a positive result.

mp-tNGS Procedures and Bioinformatics Analysis

See Supplementary Material for details of mp-tNGS procedures and bioinformatics analysis [24].

Culture of BALF and Sputum

In accordance with the standard procedure, BALF or sputum was cultured on Sabouraud's dextrose agar (LS0409, China) and incubated at 30°C. All fungal samples were incubated until they became positive or reached 14 days of incubation.

Turnaround Time for Each Test

In our center, the times of specimen submission and processing, including the time of diagnostic report generation, were all documented electronically in detail. The TAT of a

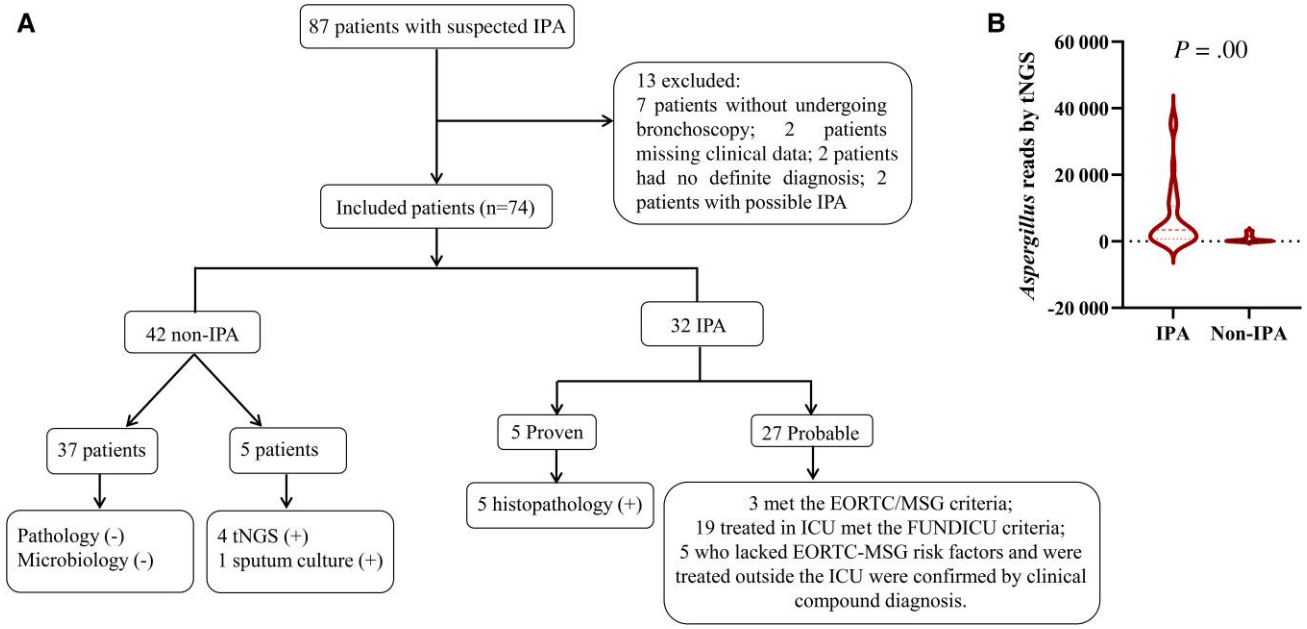

Figure 1. Study flowchart and data analysis. *A*, Flowchart of the enrollment and diagnosis of the study population. *B*, Comparison of the number of homogenized specific reads between the IPA and non-IPA groups. Abbreviations: EORTC/MSG, European Organization for the Research Treatment of Cancer Mycoses Study Group; FUNDICU, Invasive Fungal Diseases in Adult Patients in Intensive Care Unit; ICU, intensive care unit; IPA, invasive pulmonary aspergillosis; tNGS, targeted next-generation sequencing.

pathological diagnosis or laboratory test was calculated as the interval between the submission of a sample and the acquisition of a diagnostic or testing report.

Statistical Analysis

SPSS software version 26.0 was used for the statistical analysis. Normally distributed data are expressed as the mean± SDs, and *t* test was used. The measurement data are presented as percentages. Measurement data with a nonnormal distribution are expressed as the median and interquartile range, and the Mann–Whitney *U* test was used. Receiver operating characteristic (ROC) curves were designed to assess the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the estimated parameters. A χ^2 test was used to compare the diagnostic accuracy rates between the different tests. The consistency between the diagnosis of different tests and the final diagnosis was assessed by calculating a κ score. EORTC/MSG is used as the gold standard for IPA diagnosis. The positive rate and sensitivity were calculated. Probability values <5% ($P < .05$) were considered statistically significant.

RESULTS

Clinical Characteristics and Laboratory Results of Patients

Among the 87 patients with suspected IPA, 74 met the inclusion criteria and 13 were excluded. The enrolled patients were divided into IPA and non-IPA groups (Figure 1*A*) based on EORTC/MSG criteria, FUNDICU consensus, and clinical compound diagnosis. As shown in Figure 1, five of the 32 IPA patients had proven IPA according to EORTC/MSG criteria. Of the 27 patients diagnosed with probable IPA, 3 patients met the EORTC/MSG criteria, and 19 patients treated in the intensive care unit (ICU) met the FUNDICU criteria; diagnosis of the remaining 5 patients who lacked EORTC/MSG risk factors and were treated outside the ICU was based on a clinical compound diagnosis by a multidisciplinary panel of infection disease experts. As shown in Table 1, there were 56 men and 18 women, aged 31 to 83 years, with an average age of 65.2 years (SD 9.6 years). The average age of the IPA group was lower than that of the non-IPA group ($P = .02$), while no significant difference was observed in sex ($P = .51$). Overall, 42 (56.8%) and 22 (29.7%) patients had a long history of smoking and drinking, respectively; however, there was no significant difference between the 2 groups ($P = .69$, $P = .80$). Clinical symptoms included cough, expectoration, fever, etc., with no significant differences between the 2 groups ($P > .05$). The underlying diseases were diverse, and except for a history of immunosuppressants ($P = .04$), no significant differences were noted in other underlying diseases between the 2 groups.

The percentages of patients whose absolute counts of leukocytes, neutrophils, lymphocytes, platelets, and hemoglobin in peripheral blood fell below the lower limits of normal ranges were 6.8% (5/74), 4.1% (3/74), 54.1% (40/74), 14.9% (11/74), and 40.5% (30/74), respectively. However, all patients had absolute neutrophil counts above the threshold of 0.5×10^9/L. Most patients had elevated infection indicators, and percentage of

Table 1. Clinical Characteristics, Clinical Symptoms, and Laboratory Results of Enrolled Patients (n = 74)

Characteristics	Total (n = 74)	IPA (n = 32)	Non-IPA (n = 42)	χ^2 or t	P Value
Age, y, mean ± SD	65.2 ± 9.6	64.6 ± 9.9	65.7 ± 8.4	−3.80[k]	.02
Sex, male/female, No.	56/18	23/9	33/9	0.44	.51
Smoking history, ≥ 5 y, No. (%)	42 (56.8)	19 (59.4)	23 (54.8)	0.16	.69
Drinking history, ≥ 3 y, No. (%)	22 (29.7)	10 (31.3)	12 (28.6)	0.06	.80
Clinical symptoms, No. (%)					
Cough	64 (86.5)	27 (84.4)	37 (88.1)	0.22	.64
Expectoration	57 (77.0)	23 (71.9)	34 (81.0)	0.85	.36
Fever	31 (41.9)	15 (46.9)	16 (38.1)	0.58	.45
Chest distress	19 (25.7)	6 (18.8)	13 (31.0)	1.42	.23
Chest pain	10 (13.5)	3 (9.4)	7 (16.7)	0.83	.36
Dyspnea	9 (12.2)	5 (15.6)	4 (9.5)	0.63	.43
Hemoptysis	7 (9.5)	4 (12.5)	3 (7.1)	0.61	.44
Underlying disease, No. (%)					
Diabetes	15 (20.3)	9 (28.1)[a]	6 (14.3)	2.15	.14
COPD	4 (5.4)	3 (9.4)[b]	1 (2.4)	1.74	.19
Chronic liver disease	5 (6.8)	2 (6.3)[c]	3 (7.1)	0.02	.88
Interstitial lung disease	7 (9.5)	5 (15.6)[d]	2 (4.8)	2.5	.11
Solid organ malignancy	5 (6.8)	3 (9.4)[e]	2 (4.8)	0.61	.43
Obsolete pulmonary tuberculosis	14 (18.9)	5 (15.6)	9 (21.4)	0.40	.53
History of immunosuppressant	3 (4.1)	3 (9.4)[f]	0	4.1	.04
Bronchiectasis	1 (1.4)	1 (3.1)[g]	0	1.33	.25
Asthma	5 (6.8)	2 (6.3)	3 (7.1)	0.02	.88
Cardiovascular disease	28 (37.8)	13 (40.6)[h]	15 (35.7)	0.19	.67
No underlying diseases	9 (12.2)	4 (12.5)[i]	5 (11.9)	0.01	.94
Laboratory result, No. (%)					
Leukocytes, $<3.5 \times 10^9$/L	5 (6.8)	3 (9.4)	2 (4.8)	0.61	.43
Neutrophils, $<1.8 \times 10^9$/L	3 (4.1)	2 (6.3)[j]	1 (2.4)[j]	0.70	.40
Lymphocytes, $<1.1 \times 10^9$/L	40 (54.1)	22 (68.8)	18 (42.9)	4.9	.03
Platelets, $<125 \times 10^9$/L	11 (14.9)	5 (15.6)	6 (14.3)	0.03	.87
Hemoglobin, <115 g/L	30 (40.5)	18 (56.3)	12 (28.6)	5.77	.02
D-dimer, >0.25 mg/L	47 (63.5)	24 (75.0)	23 (54.8)	3.21	.07
Fibrinogen, >4 g/L	64 (86.5)	32 (100.0)	32 (76.2)	8.81	<.01
FDP, >5 mg/L	22 (29.7)	12 (37.5)	10 (23.8)	1.63	.20
Tuberculosis Ab, positive/negative	19 (25.7)	10/22	9/33	0.92	.34
ESR, >15 mm/h	58 (78.4)	27 (84.4)	31 (73.8)	0.00	1.00
IL-6, >6.6 pg/mL	40 (54.1)	28 (87.5)	12 (28.6)	0.00	1.00
Hs-CRP, >10 mg/L	54 (73.0)	26 (81.3)	28 (66.7)	0.00	1.00
Serum GM, >0.6	13 (17.6)	7 (21.9)	6 (14.3)	0.72	.40
BALF GM, >1.0	27 (36.5)	20 (62.5)	7 (16.7)	16.5	<.01
Reads of *Aspergillus* by mp-tNGS, ≥100	32 (45.1)	28 (87.5)	4 (9.5)	44.99	<.01

Abbreviations: Ab, antibody; BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; ESR, erythrocyte sedimentation rate; FDP, fibrinogen degradation products; GM, galactomannan; Hs-CRP, hypersensitive C-reactive-protein; ICU, intensive care unit; IL-6, interleukin 6; IPA, invasive pulmonary aspergillosis; mp-tNGS, multiplex targeted next-generation sequencing.

[a]The 9 patients had poorly controlled diabetes.

[b]The 3 patients had a more than 10-year history of COPD.

[c]The 2 patients had decompensated liver cirrhosis.

[d]The 5 patients had long-term low-dose glucocorticoids (<10 mg/day) treatment.

[e]Of the 3 patients, all received chemotherapy for solid organ malignancies, including 1 case of breast cancer, 1 case of malignant melanoma, 1 case of colon cancer.

[f]The 3 patients had prolonged use of corticosteroids at a mean minimum dose of 0.3 mg/kg/day of prednisone equivalent for >3 weeks, due to rheumatoid arthritis (2 cases) and connective tissue disease (1 case).

[g]Bronchiectasis complicated with hemoptysis and severe pulmonary infection treated in ICU setting.

[h]Among the 13 patients, 8 patients had hypertension, which was concurrent with other underlying diseases in Table 1, 1 patient was admitted to ICU due to viral myocarditis caused by influenza, 1 patient was admitted to ICU due to myocardial infarction combined with other underlying diseases in Table 1, and 2 patients were coronary heart disease combined with other underlying diseases in Table 1.

[i]The 4 patients did not have a clear underlying disease, but they were admitted to the ICU due to viral pneumonia, including 3 cases of COVID-19 and 1 case of influenza H1N1.

[j]Absolute counts of neutrophil $<1.8 \times 10^9$/L but $>0.5 \times 10^9$/L.

[k]means t test used; others are Chi-square test.

patients with abnormal coagulation and fibrinolysis indicators were also noted. Statistical analysis showed that the IPA group had a significantly greater proportion of patients with lymphocyte absolute counts below 1.1×10^9/L and fibrinogen levels exceeding 4 g/L than did the non-IPA group ($P = .03$, $P < .01$), while the non-IPA group had a higher incidence of patients with hemoglobin levels less than 115 g/L ($P = .02$). For the tuberculosis Ab test, 25.7% (19/74) were positive with no significant difference between the groups ($P = .34$). In the BALF GM and serum GM assays, 17.6% and 36.5% of patients exceeded the optimal cutoff value, respectively, with a significant difference in the proportion of patients with a positive BALF GM between the IPA and non-IPA groups ($P = .00$), but no significant difference was observed in the proportion of patients with a positive serum GM ($P = .40$). mp-tNGS detected specific *Aspergillus* sequences (reads per 100 000 sequencing reads ≥ 100) in 32 patients, with the proportion of patients with positive reads in the IPA group being significantly greater than that in the non-IPA group ($P < .01$). These laboratory results are presented in Table 1. Moreover, the overall mean count of reads in the IPA group was 20 503.00 (interquartile range [IQR], 8911.0, 34 709.0), which was also significantly greater than the mean count of reads of 2113.00 (IQR, 1344.00, 3027.00) in the non-IPA group ($P = 0.00$), as shown in Figure 1*B*.

Radiological and Bronchoscopic Findings

All included patients underwent a chest CT scan. The chest CT findings were mainly infectious lesions. Among 74 patients, 7 (9.5%) had a single nodule, 24 (32.4%) had multiple nodules, 40 (54.1%) had consolidations, 11 (14.9%) had cavities, 2 (2.7%) had air-crescent signs, 2 (2.7%) had halo signs, 41 (55.4%) had patchy opacity, and 3 (4.1%) had tree-in-bud signs (Supplementary Figure 1). Except for the proportion of patients with cavities ($P = .01$), there was no significant difference in chest CT findings between the 2 groups. The lesion distribution was diverse: 51.4% (38/74) in the upper lobe, 12.2% (9/74) in the middle lobe, 50.0% (37/74) in the lower lobe, and 52.7% (39/74) in multiple lobes. Except for the distribution of lesions in the upper lobe ($P = .00$), there was no significant difference between the 2 groups. Based on the radiographic indications of infection, 74 patients underwent bronchoscopy; among them, 12 presented with visible intraluminal lesions, and 62 had hyperemic and/or hypertrophic mucosa or nonspecific findings. The specific findings are detailed in Table 2, and bronchoscopic presentations are show in Supplementary Figure 2.

Table 2. Chest CT and Bronchoscopic Presentation of Enrolled Patients (n = 74)

Imaging Findings	Total (n = 74)	IPA (n = 32)	Non-IPA (n = 42)	χ^2	*P* Value
Chest CT findings, No. (%)					
Single nodule	7 (9.5)	5 (15.6)	2 (4.8)	2.50	.11
Multiple nodules	24 (32.4)	13 (40.6)	11 (26.2)	1.73	.19
Consolidations	40 (54.1)	14 (43.8)	26 (61.9)	2.41	.12
Cavities	11 (14.9)	9 (28.1)	2 (4.8)	7.83	.01
Air-crescent signs	2 (2.7)	2 (6.3)	0	2.70	.10
Halo signs	2 (2.7)	1 (3.1)	1 (2.4)	0.04	.85
Patchy opacity	41 (55.4)	16 (50.0)	25 (59.5)	0.67	.41
Tree-in-bud sign	3 (4.1)	2 (6.3)	1 (2.4)	0.70	.40
Site of lesions on chest CT, No. (%)					
Upper lobe	38 (51.4)	24 (75.0)	14 (33.3)	12.62	.00
Middle lobe	9 (12.2)	5 (15.6)	4 (9.5)	0.63	.43
Lower lobe	37 (50.0)	15 (46.9)	22 (52.4)	0.22	.64
Multiple lobes, ≥2	39 (52.7)	13 (40.6)	26 (61.9)	3.30	.07
Bronchoscopic findings, No. (%)					
Tracheobronchial ulcers	1 (1.4)	1 (3.1)	0	1.33	.25
Nodules	2 (2.7)	1 (3.1)	1 (2.4)	0.04	.85
Pseudomembranes	2 (2.7)	2 (6.3)	0	2.70	.10
Plaques	2 (2.7)	2 (6.3)	0	2.70	.10
Eschars	2 (2.7)	1 (3.1)	1 (2.4)	0.04	.85
Necrosis	3 (4.1)	1 (3.1)	2 (4.8)	0.13	.72
Hyperemic or hypertrophic mucosa	26 (35.1)	11 (34.4)	15 (35.7)	0.01	.91
Purulent secretion	19 (25.7)	7 (21.9)	12 (28.6)	0.43	.51
Nonspecific	21 (28.4)	8 (25.0)	13 (31.0)	0.32	.57

Abbreviations: CT, computed tomography; IPA, invasive pulmonary aspergillosis.

Comparison of the Detection Consistency of Serum/BALF GM, Traditional Test, and BALF tNGS

According to the EORTC/MSG, FUNDICU criteria, and clinical compound diagnosis, 32 patients were diagnosed with IPA, including 5 proven IPA (histopathological diagnosis with presence of molds) and 27 probable IPA (3 met the EORTC/MSG criteria; 19 met the FUNDICU criteria; and 5 patients with probable IPA who lacked EORTC/MSG risk factors were treated outside the ICU, and were confirmed by clinical compound diagnosis), as shown in Figure 1*A*. As shown in Table 3 and Table 4, traditional tests showed poor consistency with the final diagnosis ($\kappa = 0.442$ [SE 0.095] $P < .001$), the serum GM assay also showed poor consistency with the final diagnosis ($\kappa = 0.082$ [SE 0.098] $P = .395$), the BALF GM assay showed good consistency with the final diagnosis ($\kappa = 0.467$ [SE 0.104] $P < .001$), and mp-tNGS showed good consistency with the final diagnosis ($\kappa = 0.780$ [SE 0.074] $P < .001$). The areas under the curve (AUC) were 0.71 (95% confidence interval [CI], .58–.83; $P = .002$), 0.54 (95% CI, .40–.67; $P = .578$), 0.73 (95% CI, .61–.85; $P = .001$), and 0.89 (95% CI, .81–.97; $P = .001$), respectively (details are shown in Supplementary Figure 3).

Comparison of the Detection Performance of Serum/BALF GM, Traditional Test, and BALF mp-tNGS

Among the 74 patients, traditional diagnostic tests identified 15 cases (20.3%) of fungal infection, including 10 cases of *Aspergillus fumigatus* infection, 3 cases of *Aspergillus flavus*, 1 case of *Aspergillus niger,* and 1 case of *Aspergillus terreus*;

Table 3. Comparison of Diagnostic Consistency of Different Microbiological Methods for the Detection of *Aspergillus* (n = 74)

	Final Diagnosis		
Test Result	Positive	Negative	Total
Traditional tests			
Positive	14[a]	1[b]	15
Negative	18	41	59
Total	32	42	74
Serum GM			
Positive	7	6	13
Negative	25	36	61
Total	32	42	74
BALF GM			
Positive	20	7	27
Negative	12	35	47
Total	32	42	74
mp-tNGS			
Positive	28[c]	4[d]	32
Negative	4	38	42
Total	32	42	74

Abbreviations: BALF, bronchoalveolar lavage fluid; GM, galactomannan; mp-tNGS, multiplex targeted next-generation sequencing.

[a]Including 9 cases of *A. fumigatus*, 3 case of *Aspergillus flavus*, 1 case of *Aspergillus niger*, and 1 case of *Aspergillus terreus*.

[b]Including 1 case of *A. fumigatus*.

[c]Including 10 cases of *A. fumigatus*, 9 cases *of A. flavus*, 2 cases *of A. niger*, 5 cases *of A. fumigatum* combined with *A. flavus*, 1 case of *A. fumigatus* combined with *A. terreus*, and 1 case of *A. fumigatus* combined with *A. niger*.

[d]Including 3 cases of *A. fumigatus*, and 1 case of cases *of A. fumigatum* combined with *A. flavus*.

however, 1 case of *A. fumigatus* did not match the final diagnosis. mp-tNGS revealed fungal infection in 32 cases, including 13 cases of *A. fumigatus* infection, 9 cases *of A. flavus*, 2 cases *of A. niger*, 6 cases *of A. fumigatum* combined with *A. flavus*, 1 case of *A. fumigatus* combined with *A. terreus*, and 1 case of *A. fumigatus* combined with *A. niger*. Among the cases, the sequencing results for 3 cases of *A. fumigatus* combined with *A. flavus* infection did not match the final diagnosis, as detailed in Table 3. The sensitivity, specificity, PPV, NPV, overall diagnostic accuracy, and the average TATs of different tests for the detection of *Aspergillus* are detailed in Table 4. As shown in Figure 2, the detection sensitivity of mp-tNGS was significantly superior to that of the other tests, but no significant difference was noted in terms of specificity compared with the other tests.

Treatment and Outcome

Thirty-two patients were diagnosed with IPA and received antifungal drugs after consent. Five with proven IPA were treated per histopathology. In 27 probable IPA cases, 23 started antifungal therapy due to early GM and mp-tNGS results, and 4 based on positive lavage culture. The antifungal drugs, dosages, and courses followed 2016 Infectious Diseases Society of America guidelines [25]. Among the 32 patients, 96.9% (31/32) had symptom and/or radiological improvement. Treatment and outcomes are in Table 5.

DISCUSSION

Previous studies reported that the prevalence of IPA was predominantly confined to individuals exhibiting profound immunosuppression, including those with conditions such as neutropenia, hematological malignancies, recipients of bone marrow transplants, and those who have undergone solid organ transplantation [2, 3, 5, 10]. However, recent observations have indicated a rising incidence of IPA among individuals who do not present with severe immunodeficiency [5, 26]. Compared with neutropenic patients, the clinical manifestations of IPA in nonneutropenic patients are often atypical and easy to miss and misdiagnose, leading to delayed treatment and resulting in a higher mortality rate of IPA in nonneutropenic patients [27]. Therefore, prompt diagnosis is important for the treatment and prognosis of these patients. Currently, diagnostic methods for IPA include chest CT, bronchoscopy, conventional tests (direct microscopy/culture of BALF or sputum), GM antigen assays, and histopathology of lung biopsy, with histopathology being the gold standard for IPA diagnosis [10].

The serum GM test has demonstrated high sensitivity and specificity, exceeding 90%, in neutropenic patients [25, 28], and compared with the serum GM test, the BALF GM test achieved superior detection performance. In our study, the detection sensitivity of the BALF GM assay was in line with previous results [29]. However, the detection sensitivity of the serum GM test was 21.9%, which was slightly lower than that reported in previous studies, possibly because the cutoff value for serum GM was defined as 0.6 in our study, leading to reduced sensitivity.

Studies have reported that microscopic examination and culture of molds in BALF can achieve a specificity of up to 97%; however, the sensitivity ranges from 30% to 50% [11]. Lass-Flörl et al reported that conventional mycological culture detected invasive fungal infections with a sensitivity as low as 34% [30]. Similarly, Hoenigl et al reported a sensitivity of approximately 50% for mycological culture [31]. In our study, the sensitivity of the traditional test (direct smear of BALF/sputum, culture of BALF/sputum) was 52.3%, which was consistent with the previously reported sensitivity [11, 30, 31].

Mutcali et al conducted a multicenter study and showed that the sensitivity of the *Aspergillus* PCR in BALF was 67% (95% CI, 30%–93%), and the specificity was 98% (95% CI, 93%–99%), which is much lower than the performance of mp-tNGS in our study [32]. According to the diagnostic criteria of IPA by EORTC/MSG [8], in addition to host factors and clinical features, mycological evidence is also one of the key points in the diagnosis of probable IPA, which encompassed culture (*Aspergillus* recovered by culture of respiratory specimens) and microscopy but also indirect tests, such as antigen detection, and *Aspergillus* PCR. The essence of mp-tNGS is ultramultiplex PCR amplification of nucleic acids extracted from

Table 4. Comparison of the Sensitivity, Specificity, PPV, NPV, and Diagnostic Accuracy of Different Tests for the Detection of *Aspergillus* (n = 74)

	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)	Diagnostic Accuracy, % (95% CI)	AUC (95% CI)	TAT, Hours, Mean ± SD
Traditional test[a]	43.8 (26.4–62.3)	97.6 (87.4–99.9)	93.3 (66.0–99.0)	69.5 (62.6–75.6)	74.3 (62.8–83.8)	0.71 (.58–.83)	139.5 ± 47.8
Serum GM	21.9 (9.3–40.0)	85.7 (71.5–94.6)	53.8 (30.3–75.8)	59.0 (53.6–64.2)	58.1 (46.1–69.5)	0.54 (.40–.67)	2.80 ± 0.39
BALF GM	62.5 (43.7–78.9)	83.3 (68.6–93.0)	74.1 (58.0–85.5)	74.5 (64.6–82.3)	74.3 (62.8–83.8)	0.73 (.61–.85)	2.83 ± 0.40
mp-tNGS	87.5 (71.0–96.5)	90.5 (77.4–97.3)	87.5 (73.2–94.7)	90.5 (79.1–96.0)	89.2 (79.8–95.2)	0.89 (.81–.97)	22.10 ± 2.49

Abbreviations: AUC, area under curve; BALF, bronchoalveolar lavage fluid; CI, confidence interval; GM, galactomannan; mp-tNGS, multiplex targeted next-generation sequencing; NPV, negative predictive value; PPV, positive predictive value; TAT, turnaround time.

[a]Including direct smear of BALF/sputum, culture of BALF/sputum.

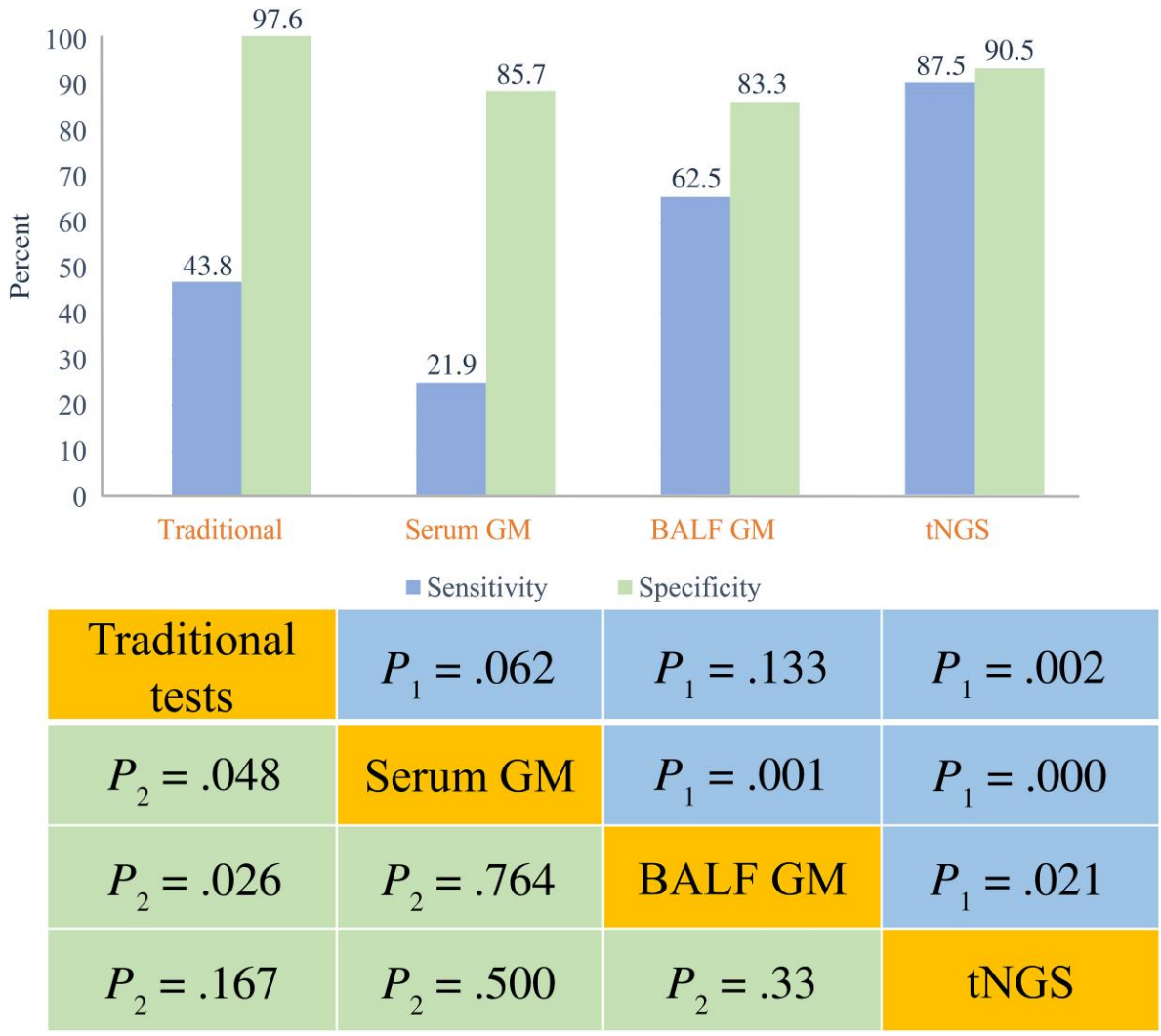

Figure 2. Comparison of the detection sensitivity and specificity among different tests for *Aspergillus*. P_1 and P_2 are *P* values for the comparison of sensitivity and specificity, respectively, between the different tests. Abbreviations: BALF, bronchoalveolar lavage fluid; GM, galactomannan; tNGS, targeted next-generation sequencing.

test samples. Therefore, the mp-tNGS method for *Aspergillus* detection has a theoretical basis and we expect mp-tNGS to be a "mycological evidence" in the revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group [8]. However, systematic evaluation of the clinical use of mp-tNGS in BALF for invasive fungal disease has been insufficient. At present, several studies

Table 5. Treatment and Outcome of Included Patients With IPA (n = 32)

Variable	Proven, No. (%) (n = 5)	Probable, No. (%) (n = 27)
The basis for initiating antifungal		
Histopathology	5 (100.0)	0
Traditional tests	0	4 (14.8)
GM antigen assay	0	5 (18.5)
mp-tNGS	0	8 (29.6)
GM antigen assay + mp-tNGS	0	10 (37.0)
Initial drug		
VCZ	1 (7.9)	3 (11.1)
VCZ + caspofungin	3 (71.1)	19 (70.4)
ESCZ	0	2 (7.4)
AmB	0	1 (3.7)
AmB + ESC/VCZ	1 (13.2)	2 (7.4)
Treatment outcome		
Failure	0	1 (3.7)
Improvement	5 (100.0)	26 (96.3)

Abbreviations: AmB, amphotericin B; ESCZ, esaconazole; GM, galactomannan; IPA, invasive pulmonary aspergillosis; mp-tNGS, multiplex targeted next-generation sequencing; VCZ, voriconazole.

have reported the application of tNGS in the detection of respiratory pathogenic microorganisms and demonstrated that tNGS has high detection sensitivity and specificity, with B. Li et al [33] confirming its rapid and accurate identification of major pathogenic *Mycobacterium* species, F. Li et al [34] reporting 87.1% detection sensitivity and 100% specificity in children with severe pneumonia, and Zhang et al [35] demonstrating over 70% concordance with clinical diagnosis and consistency with other detection methods. Furthermore, there are few studies on the detection of *Aspergillus* in BALF samples by mNGS. Zhu et al [14] and Jia et al [15] concluded that mNGS of BALF had high diagnostic efficacy for the diagnosis of IPA and was superior to traditional tests and GM assays. The study by Hu et al [36] reported that mNGS showed better performance in diagnosing pulmonary aspergillosis than culture methods did, and the combined use of reverse transcription PCR (RT-PCR) and mNGS further improved its sensitivity. However, Zhan et al [37] suggested that the combination of mNGS detection and chest CT could be used for the diagnosis of IPA in immunocompromised patients, but caution is needed in IPA diagnosis on the basis of positive mNGS results in non-immunocompromised patients. In our study, the sensitivity, specificity, and AUC of mp-tNGS for detecting *Aspergillus* in BALF samples were 87.5%, 90.5%, and 0.89, respectively, which were significant advantages over traditional assays and GM assays. The sensitivity and specificity of mp-tNGS in our study were consistent with the results of F. Li et al [34]. Moreover, compared with traditional tests, mp-tNGS shows obvious timeliness for pulmonary *Aspergillus* detection, as Vinner et al reported that tNGS has good timeliness, with a detection time of 8–16 hours [38]. Of course, mp-tNGS and other tests for quick TAT are based on the fact that we have established and are ready to use them. In the real world, the TAT is affected by many factors, such as whether the received specimens can be processed in time, whether the received specimens are qualified and the operational proficiency of laboratory staff. Therefore, the TAT of these tests in our study is a limitation. The threshold of reads per 100 000 sequencing reads for *Aspergillus* in our study is an optimal threshold obtained by our team based on the consensus of Chinese experts [39] and our clinical practice.

Considering the burden of testing costs for patients, the BALF mNGS test was not conducted in this study; thus, a comparison of detection performance between mp-tNGS and mNGS was lacking. However, as previously reported, some studies have suggested that mp-tNGS is as effective as mNGS in detecting respiratory pathogens [40, 41], whereas others have suggested that mp-tNGS is more sensitive than mNGS [42]. In addition, many studies have shown that, compared with mNGS, mp-tNGS is more cost-effective, timely, requires minimal sample quantities, and can avoid the influence of human DNA [38, 42, 43]. Therefore, the timeliness of mp-tNGS detection contributes to the prompt administration of antifungal drugs to IPA patients.

In conclusion, our study prospectively analyzed the diagnosis and treatment of 74 nonneutropenic patients with suspected IPA and preliminarily validated that BALF mp-tNGS showed good performance for the detection of pulmonary *Aspergillus* and contributions to the decision on early antifungal therapy before microbiological evidence is available. Therefore, we believe that it is worthy of clinical promotion, especially for for centers in developed countries. A shortcoming of this study was the limited number of cases, and we will collect more clinical samples for research in the future.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Data availability. The data generated in the present study are available and may be requested from the corresponding author or first author.

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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