

Original Article

Diagnostic value of targeted next-generation sequencing for distinguishing *Aspergillus* infection from colonization in non-neutropenic patients

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Abstract: Objective: To evaluate the diagnostic performance of Targeted Next-Generation Sequencing (tNGS) and determine its readout cutoff to distinguish *Aspergillus* infection from colonization in non-neutropenic patients. Methods: A total of 119 suspected patients (42 infection, 77 colonization) from 2023-2024 were enrolled per 2024 fungal consensus criteria. Among all enrolled subjects, only 52 underwent simultaneous complete testing (tNGS, culture, BDG and GM). This 52-patient subgroup was non-randomly formed because clinicians prioritized tNGS for cases with high clinical suspicion, introducing selection bias. We compared tNGS, fungal culture, BDG (≥ 70 pg/mL positive) and GM (index ≥ 0.5 positive) assays among 52 patients with complete parallel testing, and screened independent infection risk factors via logistic regression. Results: In this 52-patient subgroup, tNGS achieved an AUC of 0.912, with a sensitivity of 85.71% and a specificity of 92.31%, outperforming conventional tests. The optimal normalized read cutoff was 986; reads ≥ 986 , pleural effusion and oxygenation index < 300 were independent risk factors (all $P < 0.05$). Infection patients had higher fungal loads and lower fungal alpha diversity. Sensitivity analysis validated stable grouping results. Conclusion: tNGS enables identification of *Aspergillus* infection/colonization in non-neutropenic individuals; normalized reads of ≥ 986 provide quantitative evidence to support early antifungal intervention.

Keywords: Targeted next-generation sequencing, non-neutropenic, aspergillosis, diagnosis, colonization

Introduction

Invasive aspergillosis (IA) is a common and severe fungal infection seen in clinical practice. The disease spectrum has gradually expanded from traditionally immunocompromised individuals to non-neutropenic patients [1]. The incidence of IA is increasing annually among populations with chronic obstructive pulmonary disease, diabetes, and immune dysregulation following viral infections. These patients often present with atypical symptoms and non-specific radiological findings, leading to delayed diagnosis and persistently high mortality rates [2].

Traditional diagnostic methods have significant limitations. Fungal culture is considered the gold standard; however, its sensitivity is only

30%-50%, and obtaining results takes 1-2 weeks [3]. Microscopic examination of smears has low specificity and is easily affected by specimen contamination. Serum BDG and GM tests are influenced by antibiotics and underlying conditions, leading to high rates of false positives or false negatives [4]. Particularly in non-neutropenic patients, serological markers cannot reliably distinguish *Aspergillus* colonization from infection, often leading to overtreatment or undertreatment [5].

Targeted next-generation sequencing (tNGS) uses specific primers to capture pathogen gene sequences to achieve high-throughput sequencing, which offers advantages such as rapid detection, broad coverage, and high sensitivity [6]. Several studies have shown its excellent diagnostic performance for respiratory tract

infections. However, specific studies on *Aspergillus* infection in non-neutropenic patients remain limited, and data regarding its diagnostic efficacy and the correlation between sequence reads and infection severity are still scarce [7]. The pathogenesis of infection in non-neutropenic patients is different: mucosal barrier damage and local immune dysregulation caused by viral infection predispose to *Aspergillus* colonization and invasion [8]. Given the difficulty in distinguishing infection from colonization in such patients, highly accurate diagnostic techniques are urgently needed. This study retrospectively analyzed the clinical data of 119 suspected cases to evaluate the diagnostic value of tNGS, aiming to provide new insights and evidence for the diagnosis and treatment of this disease.

Subjects and methods

Study subjects

This retrospective study was approved by the Ethics Committee of Chifeng Municipal Hospital (Approval No.: CFYY2023008). All analysis adopted anonymized historical electronic medical records, and the ethics committee waived the requirement for written informed consent. A total of 119 patients with suspected *Aspergillus* infection admitted to Chifeng Municipal Hospital from January 1, 2023, to May 31, 2024, were retrospectively enrolled.

Notably, tNGS testing was arranged by attending clinicians based on clinical risk stratification: clinicians prioritized tNGS for patients with severe dyspnea, persistent fever, typical pulmonary CT infiltration and high suspicion of invasive aspergillosis, resulting in an unbalanced testing rate between groups: 34/42 (80.95%) patients in the infection group underwent tNGS, whereas only 18/77 (23.38%) patients in the colonization group completed tNGS. Finally, 52 patients who underwent all four tests (tNGS, fungal smear/culture, serum BDG and GM) were included for diagnostic performance comparison; this subgroup was non-randomly constituted because of clinical test-allocation bias.

Inclusion criteria: (1) Age ≥ 18 years; (2) Met any of the following conditions: ① Tested positive for *Aspergillus* by tNGS between January 1, 2023, and May 31, 2024; ② Positive for *Aspergillus* by culture of sputum or bronchoal-

veolar lavage fluid (BALF) within the same time window (January 1, 2023, to May 31, 2024); (3) Had complete clinical data, laboratory findings, and imaging data; (4) Non-neutropenic: absolute neutrophil count (ANC) $\geq 0.5 \times 10^9/L$. Exclusion criteria: (1) Neutropenia (ANC $< 0.5 \times 10^9/L$); (2) History of long-term corticosteroid therapy (prednisone > 0.3 mg/kg/day for > 3 weeks) within the past 60 days; (3) Receipt of other T-cell immunosuppressive therapy within the past 90 days; (4) Inherited immunodeficiency, stem cell transplantation, hematologic malignancy, solid organ transplantation, or presence of graft rejection; (5) Incomplete clinical data precluding diagnostic classification.

Diagnostic criteria

According to the 2024 consensus definitions of ESGCIP, EFISG, ESICM, ECOMM, MSGERC, ISAC, and ISHAM [9], patients were classified into *Aspergillus* infection and colonization groups: (1) Infection group (proven + probable), 42 cases: ① Proven cases (n=8): histopathological or cytopathological evidence of *Aspergillus* invasion in sterile site or lung tissue, or isolation of *Aspergillus* from culture of a sterile-site specimen; ② Probable cases: presence of host factors (e.g., influenza, COVID-19, moderate-to-severe COPD, decompensated cirrhosis), clinical symptoms (e.g., persistent fever, dyspnea, hemoptysis), imaging findings (e.g., pulmonary infiltrates, cavities), and at least one mycological criterion (positive fungal smear/culture, positive GM test). Notably, all patient groupings were finalized by two senior infectious disease physicians based on complete clinical data and 28-day follow-up outcomes, prior to analysis of tNGS, BDG, and GM data. Serological and sequencing indices served only as auxiliary mycological evidence for clinical diagnosis, not as grouping criteria, thereby avoiding circular diagnostic bias that might arise from overlapping test indicators. (2) Colonization group, 77 cases: *Aspergillus* detected by mycological examination but without definitive clinical evidence or imaging changes of infection; patients improved after symptomatic treatment without anti-infective therapy. (3) Note: Among host factors, a positive PCR test for influenza A/B was considered the gold standard for influenza diagnosis. In this study, three patients who tested positive only for COVID-19 antibodies (without positive viral PCR) but had typical infectious

clinical manifestations and consistent imaging/mycological findings were temporarily assigned to the infection group; the robustness of this grouping scheme was verified by sensitivity analysis in Section 2.7.

Research methods

Data collection: Clinical data were collected from the hospital's electronic medical record system, including: (1) Basic information: sex, age, underlying diseases, length of hospital stay, 28-day mortality; (2) Clinical symptoms and signs: fever, cough, dyspnea, hemoptysis, etc.; (3) Laboratory findings: procalcitonin (PCT), C-reactive protein (CRP), complete blood count, BDG test: values <70 pg/mL were considered negative, and ≥70 pg/mL positive; serum GM test: index ≥0.5 was positive, <0.5 negative; fungal smear/culture results were also recorded; (4) Imaging data: chest CT findings (e.g., nodules, cavities, air crescent sign, halo sign); (5) tNGS data: specimen type (sputum, BALF, etc.), testing time, *Aspergillus* species, and normalized sequence reads.

Testing methods: (1) Conventional mycological examination: Sputum, BALF, and other specimens were smeared and stained with Gram stain, then examined under an Olympus CX23 microscope for hyphal and spore morphology. Positive specimens were inoculated onto Sabouraud dextrose agar and incubated at 30°C for 14 days in an electric constant-temperature incubator (Shanghai Jinghong DNP-9052), with colony morphology, color, and growth rate observed daily. Species identification was performed using the bioMérieux VITEK 2 Compact automated microbiology system with fungal identification cards.

(2) BDG test: Serum specimens were collected, and (1,3)-β-D-glucan levels were measured using a kinetic turbidimetric method. The testing instrument was the Beijing Jinshanchuan Technology Development Co., Ltd. MB-80 microbial rapid kinetic detection system, with GKT-5000 kinetic fungal detection kit. Procedures were strictly followed according to the manufacturer's instructions, and values <70 pg/mL were considered negative.

(3) GM test: Serum specimens were tested for galactomannan levels using an enzyme-linked

immunosorbent assay (ELISA). The Thermo Fisher Scientific Multiskan FC automated microplate reader was used with the Zhuhai Livzon Reagent Co., Ltd. *Aspergillus* galactomannan detection kit. An index ≥0.5 was considered positive.

(4) tNGS testing: Nucleic acid was extracted from collected sputum and BALF specimens using the Qiagen QIAamp DNA Mini Kit and QIAcube automated nucleic acid extractor. The universal fungal primer panel used in this study targets full-length ITS1/ITS2 and partial 18S rRNA conserved regions, covering almost all clinically common respiratory fungi, including *Aspergillus*, *Candida*, *Mucor*, *Penicillium*, *Scedosporium* and commensal airway fungi. This primer set enables simultaneous amplification of all dominant fungal species in respiratory specimens, supporting exploratory fungal diversity analysis; however, it cannot amplify bacterial, viral, or ultra-low-abundance rare fungal sequences, and selective enrichment of fungal templates introduces inherent amplification bias. Therefore, the calculated Shannon and Simpson indices reflect only fungal community composition, rather than the complete respiratory microbiome. The target amplified regions of fungi included full-length ITS1/ITS2 internal transcribed spacer and partial 18S rRNA conserved gene fragments; specific universal fungal primers were designed to avoid cross-amplification with human host genes, with annealing temperature unified at 56°C for PCR amplification. The reference database for alignment was the NCBI RefSeq fungal ribosomal RNA standard library; the sequence-matching threshold was set as identity ≥97% and coverage ≥80% for reliable species annotation. Raw sequencing reads were filtered to obtain effective reads according to the following criteria: removal of reads with Q20 <90%, N base ratio >5%, homology to the human genome, and low-complexity repeat sequences. The normalized tNGS sequence reads were calculated as (Valid *Aspergillus* reads/Total effective microbial reads after filtering) × 100,000, which corrects for inter-sample differences in sequencing depth. Specific primers were designed targeting conserved regions of the fungal ribosomal RNA gene. PCR amplification was performed using an ABI 2720 thermal cycler. Library preparation and high-throughput sequencing were carried out on the Illumina

Table 1. Comparison of baseline clinical characteristics between the two groups [n (%), $\bar{x} \pm s/M$ (Q1, Q3)]

Characteristic	Infection group (n=42)	Colonization group (n=77)	$\chi^2/t/Z$	P
Sex (male)	29 (69.05)	47 (61.04)	0.755	0.385
Age (years)	67.52±11.63	65.41±12.54	0.899	0.370
Hospital stays (days)	29.38 (19.51, 40.26)	19.62 (12.38, 28.74)	4.126	<0.001
28-day mortality	15 (35.71)	4 (5.19)	18.866	<0.001
Underlying diseases				
COPD	23 (54.76)	15 (19.48)	15.564	<0.001
Diabetes mellitus	13 (30.95)	14 (18.18)	2.527	0.112
Hypertension	16 (38.10)	25 (32.47)	0.381	0.537
COVID-19 infection	17 (40.48)	6 (7.79)	18.619	<0.001
Solid tumor	5 (11.90)	7 (9.09)	0.237	0.626
Liver cirrhosis	4 (9.52)	4 (5.19)	0.812	0.367

Notes: COPD, Chronic Obstructive Pulmonary Disease; COVID-19, Coronavirus Disease 2019.

NovaSeq 6000 sequencing platform. Sequencing data were analyzed bioinformatically, aligned with reference sequences of *Aspergillus*, and the tNGS normalized sequence reads were calculated as the number of *Aspergillus* sequences per 100,000 effective sequencing reads, which corrects for differences in sequencing depth between samples.

Statistical methods

SPSS 26.0 statistical software was used for data analysis. Normally distributed continuous variables were expressed as mean $\pm$ standard deviation, and comparisons between groups were performed using the t-test. Non-normally distributed data were expressed as median (interquartile range) [M (Q1, Q3)], and comparisons between groups were performed using the Mann-Whitney U test. Categorical variables were expressed as number (percentage) [n (%)]. For contingency table analysis: when total sample size ≥ 40 and all expected frequencies ≥ 5 , the Pearson χ^2 test was adopted; when total sample size ≥ 40 but at least one expected frequency was between 1 and 5, the continuity-corrected χ^2 test was applied; when total sample < 40 or any expected frequency < 1 , Fisher's exact test was performed. The diagnostic performance of each testing method was analyzed using receiver operating characteristic (ROC) curves, and the area under the curve (AUC), sensitivity, specificity, positive predictive value, and negative predictive value were calculated. Multivariate logistic regression analysis was used to identify independent risk factors for *Aspergillus* infection. A P-value of < 0.05 was considered statistically significant.

Results

Basic clinical characteristics of patients

Of the 119 patients, 76 (63.87%) were male and 43 (36.13%) were female. Age ranged from 28 to 97 years, with a mean of 65.80 ± 12.30 years. Hospital stays ranged from 3 to 79 days, with a median of 21.45 (14.23, 32.67) days. Nineteen patients (15.97%) died within 28 days. Regarding underlying diseases, 41 patients had hypertension (34.45%), 38 had COPD (31.93%), 27 had diabetes mellitus (22.69%), 23 had COVID-19 infection (19.33%), and 12 had solid tumors (10.08%). There were 42 cases in the infection group and 77 in the colonization group. No significant differences were observed between the two groups in sex, age, history of diabetes mellitus, history of hypertension, history of solid tumor, or history of liver cirrhosis ($P > 0.05$). Compared with the colonization group, the infection group had significantly longer hospital stays, higher proportions of COPD and COVID-19 infection, and a higher 28-day mortality rate ($P < 0.05$) (Table 1).

Comparison of laboratory findings

Compared with the colonization group, the infection group had significantly higher PCT, CRP, WBC count, and absolute neutrophil count, and a significantly lower absolute lymphocyte count ($P < 0.05$). The oxygenation index in the infection group [226.74 (168.31, 274.90)] was markedly lower than that in the colonization group [358.26 (312.47, 401.53)] ($Z = 6.742$, $P < 0.05$). The positivity rates of the BDG test

Table 2. Comparison of laboratory findings between the two groups [M (Q1, Q3)/ $\bar{x} \pm s$, n (%)]

Indicator	Infection group (n=42)	Colonization group (n=77)	Z/t/ χ^2	P
PCT (ng/L)	0.72 (0.18, 1.95)	0.11 (0.05, 0.32)	5.124	<0.001
CRP (mg/L)	92.34±41.82	33.51±27.93	9.166	<0.001
White blood cell count ($\times 10^9/L$)	11.53±4.62	9.64±3.51	2.504	0.014
Absolute neutrophil count ($\times 10^9/L$)	8.92±4.03	7.43±3.02	2.279	0.024
Absolute lymphocyte count ($\times 10^9/L$)	0.85±0.40	1.42±0.56	5.830	<0.001
Oxygenation index	226.74 (168.31, 274.90)	358.26 (312.47, 401.53)	6.742	<0.001
Hemoglobin (g/L)	116.32±21.45	128.67±17.32	3.412	0.001
Platelet count ($\times 10^9/L$)	207.45±86.32	248.76±93.45	2.366	0.020
BDG test positive	20 (47.62)	8 (10.39)	20.934	<0.001
GM test positive	22 (52.38)	5 (6.49)	32.623	<0.001
BALF-GM positive	4 (9.52)	0 (0.00)	7.588	0.006

Notes: BDG positivity was defined as ≥ 70 pg/mL; serum GM positivity as index ≥ 0.5 . The table presents only categorical positive/negative counts. PCT, Procalcitonin; CRP, C-Reactive Protein; ANC, Absolute Neutrophil Count; BDG, 1,3- β -D-glucan.

Table 3. Comparison of chest CT findings between the two groups [n (%)]

Imaging finding	Infection group (n=42)	Colonization group (n=77)	χ^2	P
Nodular shadow	28 (66.67)	41 (53.25)	2.009	0.156
Cavitation	25 (59.52)	6 (7.79)	37.753	<0.001
Consolidation	33 (78.57)	31 (40.26)	16.047	<0.001
Patchy ground-glass opacity	37 (88.10)	46 (59.74)	10.355	0.001
Halo sign	7 (16.67)	0 (0.00)	13.635	<0.001
Pleural effusion	21 (50.00)	9 (11.69)	22.318	<0.001
Air crescent sign	5 (11.90)	0 (0.00)	9.561	0.002

(defined as BDG ≥ 70 pg/mL) and serum BALF-GM/GM test (defined as GM index ≥ 0.5) were all significantly higher in the infection group than in the colonization group ($P < 0.05$) (**Table 2**).

Comparison of imaging findings

The incidences of cavitation, consolidation, patchy ground-glass opacity, and pleural effusion were significantly higher in the infection group; the air crescent sign was observed only in the infection group (**Table 3**).

tNGS detection and microbial diversity analysis

A total of 52 patients underwent tNGS testing (34 in the infection group and 18 in the colonization group). The specimen type distribution among the 52 patients was 31 BALF specimens (59.62%) and 21 sputum specimens (40.38%). In the infection group, there were 22 BALF and 12 sputum; in the colonization group, 9 BALF and 9 sputum. Subgroup comparison showed

that normalized *Aspergillus* sequence reads in BALF specimens [876.52 (421.37, 1642.90)] were significantly higher than those in sputum specimens [412.65 (189.22, 763.14)] ($P < 0.05$) (**Table 4**).

Various *Aspergillus* species were detected in these 52 patients; all counts in **Table 5** are patient-based (each patient counted once). Mixed infection refers to a single specimen from one patient testing positive for two or more distinct *Aspergillus* species simultaneously, rather than separate independent pathogen episodes. Therefore, the sum of individual species counts may exceed the total number of patients, but no patient was counted twice. Species included *Aspergillus fumigatus* and *Aspergillus flavus* complex. The normalized tNGS sequence reads in the infection group [1286.35 (854.62, 2135.78)] were significantly higher than those in the colonization group [327.41 (156.89, 589.33)] ($P < 0.05$). Exploratory fungal community diversity analysis, limited to primer-captured fungal taxa, showed that

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Table 4. Distribution of specimen types among 52 patients with complete tNGS and auxiliary tests [n (%)]

Specimen type	Total (n=52)	Infection group (n=34)	Colonization group (n=18)	χ^2	P
BALF	31 (59.62)	22 (64.71)	9 (50.00)	1.012	0.314
Sputum	21 (40.38)	12 (35.29)	9 (50.00)		

Note: BALF, Bronchoalveolar Lavage Fluid.

Table 5. Comparison of tNGS detection and microbial diversity indices [n (%), M (Q1, Q3)]

Indicator	Infection group (n=34)	Colonization group (n=18)	χ^2/Z	P
<i>Aspergillus</i> species				
<i>Aspergillus fumigatus</i>	20 (58.82)	11 (61.11)	0.026	0.873
<i>Aspergillus flavus</i> complex	7 (20.59)	5 (27.78)	0.343	0.558
<i>Aspergillus niger</i> complex	4 (11.76)	0 (0.00)	2.294	0.130
<i>Aspergillus terreus</i> complex	1 (2.94)	0 (0.00)	0.540	0.463
<i>Aspergillus versicolor</i>	0 (0.00)	0 (0.00)	0.000	1.000
Mixed infection	2 (5.88)	2 (11.11)	0.453	0.501
Normalized sequence reads	1286.35 (854.62, 2135.78)	327.41 (156.89, 589.33)	6.213	<0.001
Shannon index	1.38 (0.92, 1.85)	2.16 (1.63, 2.74)	3.426	0.001
Simpson index	0.62 (0.45, 0.78)	0.35 (0.23, 0.48)	3.317	0.001

Notes: Statistical unit is individual patient (infection group n=34, colonization group n=18). Mixed infection means a single specimen simultaneously detected ≥ 2 *Aspergillus* species in one patient, each pathogen detection count is descriptive statistical value, so the sum of each species detection number exceeds the total number of patients in the group, without repeated counting of individual patients. Shannon index reflects fungal alpha diversity; Simpson index reflects fungal dominance.

the Shannon index in the infection group [1.38 (0.92, 1.85)] was lower than that in the colonization group [2.16 (1.63, 2.74)], while the Simpson index [0.62 (0.45, 0.78)] was higher than that in the colonization group [0.35 (0.23, 0.48)] ($P<0.05$) (Table 5). These diversity indices reflect only fungal composition and cannot represent the full respiratory microbiome because of the selective amplification bias inherent to tNGS (Table 5).

Comparison of diagnostic performance of different testing methods

All diagnostic performance metrics presented below are derived exclusively from the 52-patient subgroup with complete parallel testing and cannot be generalized to the full cohort of 119 suspected *Aspergillus* patients. Within this limited subgroup, the AUC for tNGS was 0.912 (95% CI: 0.856-0.968), with a sensitivity of 85.71% and a specificity of 92.31%, outperforming conventional mycological examination, BDG and GM tests in this subset only (Table 6).

Risk factors for *Aspergillus* infection

Univariate analysis showed that COPD, COVID-19, pleural effusion, PCT >0.5 ng/L, CRP >50

mg/L, absolute lymphocyte count $<1.1 \times 10^9/L$, BDG positivity, GM positivity, oxygenation index <300 , and normalized tNGS sequence reads ≥ 986 were associated with *Aspergillus* infection (all $P<0.05$). Multivariate logistic regression analysis showed that normalized tNGS sequence reads ≥ 986 (OR=8.790, 95% CI: 3.021-25.628, $P<0.05$), pleural effusion (OR=3.941, 95% CI: 1.332-11.659, $P<0.05$), and oxygenation index <300 (OR=4.281, 95% CI: 1.491-12.287, $P<0.05$) were independent risk factors for *Aspergillus* infection. Detailed regression data are presented in Table 7.

Sensitivity analysis for grouping robustness

Three patients in the original infection cohort were COVID-19 antibody-positive only, without nucleic acid PCR confirmation. To verify whether their temporary inclusion affected the study conclusions, we performed a sensitivity analysis by reassigning these three patients to the colonization group and repeating all between-group comparisons, logistic regression and ROC diagnostic performance calculations. ① After reclassification, the revised infection group contained 39 patients and the colonization group contained 80 patients. The inter-group differences in hospital stay, 28-day mor-

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Table 6. Comparison of diagnostic performance and turnaround time among different testing methods (analysis restricted to the 52 patients with complete simultaneous testing, not applicable to the full enrolled cohort)

Testing method	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	Positive predictive value (%) (95% CI)	Negative predictive value (%) (95% CI)	AUC (95% CI)	Turnaround time (x ± s, h)
tNGS (n=52)	85.71 (69.72-95.18)	92.31 (80.77-97.68)	83.33 (66.24-93.61)	94.12 (83.79-98.64)	0.912 (0.856-0.968)	23.50±3.20
Conventional mycological examination (n=52)	52.38 (33.85-70.98)	87.18 (73.68-95.12)	68.75 (49.23-84.06)	76.19 (63.29-86.42)	0.697 (0.582-0.812)	142.80±45.60
BDG test (n=52)	47.62 (29.29-66.50)	84.62 (70.60-93.21)	62.50 (43.66-79.08)	74.36 (61.89-84.60)	0.654 (0.531-0.777)	2.80±0.40
GM test (n=52)	50.00 (31.89-68.11)	83.33 (69.09-92.30)	63.64 (44.19-80.07)	73.53 (60.79-83.89)	0.665 (0.543-0.787)	2.90±0.50

Notes: All ROC-derived AUC, sensitivity, specificity, PPV and NPV values were calculated solely from the 52-patient subgroup with complete tNGS, culture, BDG and GM results; these parameters cannot be extrapolated to the overall 119 suspected *Aspergillus* population due to non-random subgroup selection bias. tNGS, Targeted Next-Generation Sequencing.

Table 7. Multivariate logistic regression analysis of *Aspergillus* infection

Variable	β	S.E	Z	P	OR (95% CI)
tNGS normalized sequence reads ≥ 986	2.175	0.552	15.080	<0.001	8.790 (3.021-25.628)
Pleural effusion	1.372	0.516	7.080	0.013	3.941 (1.332-11.659)
Oxygenation index <300	1.453	0.507	8.260	0.007	4.281 (1.491-12.287)
COPD	0.925	0.541	2.927	0.087	2.522 (0.903-7.035)
GM test positive	0.897	0.547	2.723	0.099	2.452 (0.859-6.998)

Notes: COPD, Chronic Obstructive Pulmonary Disease; GM, Galactomannan.

tality, prevalence of COPD/COVID-19, inflammatory markers, oxygenation index, chest CT findings, and normalized tNGS reads remained statistically significant (all $P < 0.05$), consistent with the primary results. ② Among the 52 patients with complete tests, removing the three antibody-only cases did not change the tNGS ROC cutoff value (986); AUC, sensitivity and specificity only fluctuated within 0.01 and 1%, without substantive change. ③ Multivariate logistic regression still identified normalized tNGS reads ≥ 986 , pleural effusion and oxygenation index <300 as independent risk factors, with ORs and 95% CIs showing no obvious deviation from the original model. The above sensitivity analysis confirmed that the temporary inclusion of COVID-19 antibody-positive patients did not alter the core study outcomes, demonstrating the robustness of the original grouping strategy.

Discussion

This study focused on distinguishing *Aspergillus* infection from colonization in non-neutropenic patients. tNGS exhibited better diagnostic efficiency than traditional tests only within the 52 clinically selected non-random subgroup, with an optimal cutoff of normalized reads at 986. Multivariate regression confirmed tNGS reads ≥ 986 , pleural effusion, and oxygenation index <300 as independent risk factors. The elevated normalized reads in infection patients are attributable to invasive fungal proliferation and local inflammatory lysis. BALF specimens carry higher fungal load signals than sputum, which may interfere with quantitative assessment. The fungal diversity analysis is exploratory with inherent tNGS amplification bias and cannot reflect overall respiratory microbiome. Sensitivity analysis verified the reliability of grouping standards. This study is limited by its single-center retrospective design and non-random selection bias in the 52-patient subgroup;

therefore, the diagnostic results cannot be extrapolated to all suspected patients. Future multicenter prospective trials with unified tNGS testing are needed to further verify our findings.

The incidence of *Aspergillus* infection in non-neutropenic patients is increasing annually, and underlying conditions such as COPD, diabetes, and post-viral immune dysregulation are important predisposing factors [10]. In these patients, immune function is not completely compromised; *Aspergillus* infection usually presents as locally invasive disease, symptoms overlap with the underlying condition, and imaging findings lack specificity. Traditional diagnostic methods are inadequate for early identification [11]. Fungal culture is the gold standard but has a sensitivity of only 30%-50% and takes 1-2 weeks to obtain results, which can delay treatment [12]. BDG and GM assays are susceptible to interference by antibiotic use and underlying diseases, resulting in persistently high false positive and false negative rates, and their utility in distinguishing colonization from infection is limited [13]. tNGS eliminates the need for pathogen culture by directly detecting pathogen nucleic acids through targeting conserved regions of fungal ribosomal RNA genes. Its turnover time is only 23.50 ± 3.20 hours, much shorter than the 142.80 ± 45.60 hours required for traditional culture, thus meeting the need for early clinical intervention [14].

Only in the non-random subgroup of 52 patients who underwent all tests in parallel did tNGS show a sensitivity of 85.71%, a specificity of 92.31%, and an AUC of 0.912, outperforming conventional mycological examination, BDG, and GM in this subgroup. This advantage cannot be generalized to all 119 enrolled suspected patients because of subgroup selection bias. This is closely related to the technical

advantages of tNGS, which can effectively enrich low concentrations of pathogen nucleic acids and reduce the interference of host and other microbial genes [15].

Normalized tNGS sequence reads were significantly higher in the infected group than in the colonized group. The optimal cutoff value determined by ROC curve analysis was 986. Multiple logistic regression confirmed that normalized reads ≥ 986 was an independent risk factor for infection ($P < 0.05$). The underlying mechanism can be explained by the pathogenesis of *Aspergillus* SPP. In the colonization state, *Aspergillus* only attaches to the respiratory mucosa and does not invade the lung parenchyma, resulting in slow fungal proliferation and limited release of nucleic acids. In contrast, during infection, *Aspergillus* secretes proteases, phospholipases, and other enzymes that disrupt the host mucosal barrier. After invading lung tissue, the fungus proliferates extensively, leading to a marked increase in pathogen nucleic acid concentration in the specimen and a corresponding rise in sequencing read counts [16]. In addition, the local inflammatory response triggered by infection further promotes fungal lysis and nucleic acid release, forming a positive feedback loop of “infection-inflammation-increased nucleic acid release”. Thus, tNGS read counts can objectively reflect the invasive capacity and infectious burden of the pathogen [17]. In addition, we observed significant differences in normalized sequence reads between BALF and sputum samples. Bronchoscopically collected BALF allows direct sampling of alveolar lesions, reducing upper airway bacterial contamination and yielding higher *Aspergillus* read abundance, whereas sputum is susceptible to oropharyngeal *Aspergillus* colonization, which may partially explain the greater variability in sequence counts from sputum samples. We also performed an exploratory fungal diversity analysis using the tNGS universal fungal primer panel. The decreased Shannon index and increased Simpson index in infected patients indicated the dominance of *Aspergillus* in the fungal community. However, this analysis has inherent technical limitations: tNGS enriches only fungal templates and excludes bacteria and viruses; therefore, diversity metrics cannot characterize the complete respiratory microbiome. Consequently, these results should be

interpreted cautiously as preliminary exploratory findings. In addition, microbial diversity analysis showed that compared with the colonized group, the infected group had a lower Shannon index and a higher Simpson index, indicating that the imbalance of respiratory microbiota was more obvious in the infected state, and *Aspergillus* became the dominant bacteria. This finding is consistent with previous reports that fungal infections are accompanied by a reduction in microbial diversity [18].

This study identified pleural effusion and oxygenation index < 300 as independent risk factors for *Aspergillus* infection (both $P < 0.05$), which were directly related to the pathophysiology of invasive aspergillosis. The inflammatory response caused by *Aspergillus* invasion of the lung parenchyma increases alveolar capillary permeability, leading to accumulation of inflammatory exudates and pleural effusion. Simultaneously, lung tissue destruction and impaired alveolar ventilation directly lead to a decreased oxygenation index [19]. Moreover, the proportions of COPD and COVID-19 were significantly higher in the infection group than in the colonization group, suggesting that these conditions impair the respiratory mucosal barrier and local immune regulation, rendering patients more susceptible to invasive aspergillosis. This is consistent with the core host factors for *Aspergillus* infection in non-neutropenic patients clearly outlined in the 2024 FUNDICU consensus [20]. We also performed sensitivity analyses to verify group stability. After reclassifying the three COVID-19 antibody-positive, PCR-negative patients from the infection group to the colonization group, all between-group comparisons, ROC diagnostic metrics, and multivariate logistic regression results showed no significant deviations from the primary analysis, indicating that our initial grouping criteria were robust and that the core conclusions were not biased by these three boundary cases.

Regardless of the extensive work done here, this study still has several limitations. (1) As a single-center retrospective study, the sample size is limited and selection bias may exist, especially in the non-random composition of the 52-patient subgroup for diagnostic performance analysis. Clinicians preferentially ordered tNGS for patients with a high clinical

suspicion of invasive aspergillosis, resulting in an obvious disparity in tNGS testing rate between infection group (80.95%) and colonization group (23.38%). This uneven allocation may artificially elevate the specificity and negative predictive value of tNGS in the ROC analysis; therefore, the diagnostic parameters in this study should be interpreted with caution and cannot be directly extrapolated to all suspected *Aspergillus* populations without a uniform testing strategy. In addition, only 8 out of the 42 infected patients were histopathologically proven to have IA, whereas the remaining 34 were clinically probable cases diagnosed by composite clinical and mycological criteria. Although grouping was completed before analyzing tNGS and serological results to avoid circular bias, reliance on clinical composite criteria may introduce some diagnostic confounding; prospective studies with more pathologically confirmed cases are needed to validate our findings. All diagnostic performance indicators of tNGS were only analyzed in a non-random 52-patient subgroup, and the conclusion that tNGS is superior to conventional assays is limited to this subset and cannot be extended to the full cohort of 119 patients with suspected *Aspergillus* infection. (2) Specimen type affects normalized sequence read counts; BALF samples carry higher fungal load signals than sputum, which may confound quantitative interpretation. Stratified analysis by specimen type is warranted in future large-sample studies. (3) The Shannon and Simpson diversity indices in this study are exploratory and are limited to fungal communities captured by the tNGS universal fungal primers. The tNGS platform selectively amplifies fungal nucleic acids and cannot cover bacterial, viral, or ultra-low-abundance microbial components in respiratory samples, introducing inherent amplification bias. Therefore, the observed fungal community differences cannot represent overall airway microbiome disturbance, and further untargeted mNGS with unbiased whole-microbe amplification is required to verify these changes. (4) This study did not evaluate the utility of tNGS for detecting *Aspergillus* resistance genes, although resistance monitoring is critical for guiding antifungal therapy. Future multicenter prospective studies with larger sample sizes and incorporation of resistance gene analysis are warranted to further refine the clinical utility of tNGS in the diagnosis and management of *Aspergillus* infection.

In conclusion, tNGS offers the advantages of high sensitivity, good specificity, and rapid turnaround in the diagnosing *Aspergillus* infection in non-neutropenic patients. The normalized tNGS sequence read count, with an ROC-validated cutoff of ≥ 986 , can effectively distinguish infection from colonization. When combined with clinical indicators such as pleural effusion and oxygenation index < 300 , tNGS may further improve diagnostic accuracy, providing a reliable basis for early and precise initiation of antifungal therapy. Therefore, its application is worthy of further promotion.

Disclosure of conflict of interest

None.

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References

- [1] Ito Y, Takazono T, Yura H, Yoshida M, Takeda K, Ide S, Iwanaga N, Sakamoto N, Yanagihara K and Mukae H. Clinical characteristics and prognosis of pulmonary aspergillosis complicating interstitial lung diseases. *Respir Investig* 2025; 63: 1293-1299.
- [2] Kim T and Choi SH. Diabetes mellitus and infectious diseases: current evidence and clinical implications. *Diabetes Metab J* 2025; 49: 915-933.
- [3] Yu YY, Meng XH and Liu Y. Comparison of the diagnostic value of GM test, microbial culture, and mNGS for COPD complicated with fungal infection and their significance in guiding clinical treatment. *Lab Med Clin* 2025; 22: 3264-3268.
- [4] Cruciani M, White PL, Mengoli C, Löffler J, Morton CO, Klingspor L, Buchheidt D, Maertens J, Heinz WJ, Rogers TR, Weinbergerova B, Warris A, Lockhart DEA, Jones B, Cordonnier C, Donnelly JP and Barnes RA; Fungal PCR Initiative. Research progress on causes of false-positive galactomannan test in the diagnosis of invasive aspergillosis. *Chin J Tuberc Respir Dis* 2020; 43: 862-866.
- [5] Deng J and Li L. Interpretation of expert consensus on the diagnosis, treatment and management of chronic obstructive pulmonary disease with pulmonary aspergillosis. *Chin J New Clin Med* 2025; 18: 120-124.
- [6] Chang Z, Deng J, Zhang J, Wu H, Wu Y, Bin L, Li D, Liu J, Yu R, Lin H, An L and Sun B. Rapid and

- accurate diagnosis of urinary tract infections using targeted next-generation sequencing: a multicenter comparative study with metagenomic sequencing and traditional culture methods. *J Infect* 2025; 90: 106459.
- [7] Ren TC and Wang HS. Diagnosis of non-neutropenic invasive pulmonary aspergillosis by targeted next-generation sequencing of bronchoalveolar lavage fluid combined with galactomannan detection. *J Hubei Univ Med* 2024; 43: 640-643.
- [8] Wang Z, Schmit T, Guo K, Tripathi JK, Navaeisiddighi Z, Tyagi A, Mathur R, Hur J, Jurivich D and Khan N. Loss of pulmonary tissue protection and neutrophil microbicidal defects promote severe *Aspergillus fumigatus* infection during influenza A virus infection. *Infect Immun* 2025; 93: e0023425.
- [9] Bassetti M, Giacobbe DR, Agvald-Ohman C, Akova M, Alastruey-Izquierdo A, Arikan-Akdagli S, Azoulay E, Blot S, Cornely OA, Cuenca-Estrella M, de Lange DW, De Rosa FG, De Waele JJ, Dimopoulos G, Garnacho-Montero J, Hoenigl M, Kanj SS, Koehler P, Kullberg BJ, Lamoth F, Lass-Flörl C, Maertens J, Martin-Loeches I, Muñoz P, Poulakou G, Rello J, Sanguinetti M, Taccone FS, Timsit JF, Torres A, Vazquez JA, Wauters J, Asperges E, Cortegiani A, Grecchi C, Karaikos I, Le Bihan C, Mercier T, Mortensen KL, Peghin M, Rebuffi C, Tejada S, Vena A, Zuccaro V, Scudeller L and Calandra T; Study Group for Infections in Critically Ill Patients of the European Society of Clinical Microbiology and Infectious Diseases (ESGCI), the Fungal Infection Study Group of the European Society of Clinical Microbiology and Infectious Diseases (EFISG), the European Society of Intensive Care Medicine (ESICM), the European Confederation of Medical Mycology (ECMM), the Mycoses Study Group Education and Research Consortium (MSGERC), the International Society of Antimicrobial Chemotherapy (ISAC), the International Society for Human and Animal Mycology (ISHAM), the Austrian Society for Medical Mycology (ÖGMM), the Italian Society of Anesthesia, Analgesia, Reanimation, and Intensive Care (SIAARTI), the Italian Society of Anti-Infective Therapy (SITA), and the FUNDICU Collaborators. Invasive fungal diseases in adult patients in intensive care unit (FUNDICU): 2024 consensus definitions from ESGCI, EFISG, ESICM, ECMM, MSGERC, ISAC, and ISHAM. *Intensive Care Med* 2024; 50: 502-515.
- [10] Chinese Thoracic Society of Chinese Medical Association. Expert consensus on the diagnosis, treatment and management of chronic obstructive pulmonary disease with pulmonary aspergillosis. *Zhonghua Jie He He Hu Xi Za Zhi* 2024; 47: 604-622.
- [11] Wang X, Lu Y, Sun C, Zhong H, Cai Y, Cao M, Cui X, Sun W, Wang L, Lu X, Chen C, Chen Y, Feng C, Tao Y, Zhou J, Shi J, Ma G, Li Y and Su X. Development and validation of a machine learning-based diagnostic model for identifying nonneutropenic invasive pulmonary aspergillosis in suspected patients: a multicenter cohort study. *Microbiol Spectr* 2025; 13: e0060725.
- [12] Wu F, Tian J, She Z, Liu Y, Wan W and Wen C. Diagnosis of pulmonary mucormycosis in hematological patients by pathogen mNGS and *Mucorales* PCR. *Lab Med* 2024; 39: 1145-1149.
- [13] Wei Z, Li S, Zhu J and Wang H. Assessment of the 1,3-β-D-glucan test and the galactomannan antigen test in the detection of invasive fungal infections in patients with hematological diseases. *Microbiol Spectr* 2025; 13: e0120925.
- [14] Wang H, Chen X, You H, Wang Y, Xia X, Tang Y, Ren T and Wang M. Performance of mp-tNGS in bronchoalveolar lavage fluid for the diagnosis of invasive pulmonary aspergillosis in non-neutropenic patients. *J Infect Dis* 2025; 231: 1609-1618.
- [15] Yang C, Gao W, Guo Y and Zeng Y. Nanopore-based targeted next-generation sequencing (tNGS): a versatile technology specialized in detecting low bacterial load clinical specimens. *PLoS One* 2025; 20: e0324003.
- [16] Jiang Z, Gai W, Zhang X, Zheng Y, Jin X, Han Z, Ao G, He J, Shu D, Liu X, Zhou Y and Hua Z. Clinical performance of metagenomic next-generation sequencing for diagnosis of pulmonary *Aspergillus* infection and colonization. *Front Cell Infect Microbiol* 2024; 14: 1345706.
- [17] Liu DL, Wang J and Guan WD. Joint research on quality evaluation of targeted next-generation sequencing technology. *China Food Drug Adm Mag* 2025; 3: 92-103.
- [18] Seear PJ, Welsh KG, Satchwell J, Patel D, Pashley CH, Wardlaw AJ and Gaillard EA. Positive sputum fungal culture, fungal sensitisation, and airway microbial diversity in asthmatic children. *Med Mycol* 2025; 63: myaf005.
- [19] Odeyemi J, Emoto J and Abu-Samra AG. Pyopneumothorax caused by *Aspergillus terreus* and *Haemophilus influenzae*: a case report. *Cureus* 2024; 16: e76683.
- [20] Liu JH, Chen DS and Li Z. Research advances in diagnosis and treatment of influenza virus and SARS-CoV-2 associated invasive pulmonary aspergillosis. *Acad J Chin PLA Med Sch* 2023; 44: 1151-1156.