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Bronchoalveolar lavage fluid metagenomic next-generation sequencing assay for identifying pathogens in lung cancer patients

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Key words: Lung cancer, Bronchoalveolar lavage fluid, Metagenomic next-generation sequencing, Infectious diseases

Abstract: Background: For patients with lung cancer, timely identification of new lung lesions as infectious or non-infectious, and accurate identification of pathogens is very important in improving OS of patients. As a new auxiliary examination, metagenomic next-generation sequencing (mNGS) is believed to be more accurate in diagnosing infectious diseases in patients without underlying diseases, compared with conventional microbial tests (CMTs). We designed this study to find out whether mNGS has better performance in distinguishing infectious and non-infectious diseases in lung cancer patients using bronchoalveolar lavage fluid (BALF). **Materials and Methods:** This study was a real-world retrospective review based on electronic medical records of lung cancer patients with bronchoalveolar lavage (BAL) and BALF commercial mNGS testing as part of clinical care from 1 April 2019 through 30 April 2022 at The First Affiliated Hospital of Sun Yat-sen University. 164 patients were included in this study. Patients were categorized into the pulmonary non-infectious disease (PNID) group (n = 64) and the pulmonary infectious disease (PID) group (n = 100) groups based on final diagnoses. **Results:** BALF mNGS increased the sensitivity rate by 60% compared to CMTs (81% *vs.* 21%, $p < 0.05$), whereas there was no significant difference in specificity (75% *vs.* 98.4%, $p > 0.1$). Among the patients with PID, bacteria were the most common cause of infection. Fungal infections occurred in 32% of patients, and *Pneumocystis Yersini* was most common. Patients with Tyrosine kinase inhibitors (TKIs) therapy possess longer overall survival (OS) than other anti-cancer agents, the difference between TKIs and immuno-checkpoint inhibitors (ICIs) was insignificant (median OS TKIs *vs.* ICIs *vs.* Anti-angiogenic *vs.* Chemo *vs.* Radiotherapy = 76 *vs.* 84 *vs.* 61 *vs.* 58 *vs.* 60). **Conclusions:** our study indicates that BALF mNGS can add value by improving overall sensitivity in lung cancer patients with potential pulmonary infection, and was outstanding in identifying Pneumocystis infection. It could be able to help physicians adjust the follow-up treatment to avoid the abuse of antibiotics.

Introduction

The non-specific symptoms, such as cough, fever, and dyspnea, can appear in both infectious and non-infectious diseases of pulmonary, including lung cancer. It is difficult to distinguish between pulmonary infectious disease (PID) and pulmonary non-infectious disease (PNID) according to these non-specific symptoms discovered by inquiry and physical examination alone [1]. Patients with lung cancer are often vulnerable to infection, and the risk is increased by tumor-associated immunosuppression and the effects of the treatments [2,3]. Infection negatively affects clinical outcomes and overall survival (OS) [4]; timely identification of PID/PNID, pathogens, and follow-up adjustment of antibiotics are very important for the management of lung cancer patients with new-onset infection-like symptoms and findings on computed tomography (CT) image [5,6].

Anti-cancer treatments can cause PNID, including radiation and interstitial pneumonitis [7], which calls for totally different management compared to pneumonia and other PIDs. When infectious diseases are mistaken for non-infectious diseases, the interruption of antibiotics treatment and the administration of immunosuppressive agents such as steroids can lead to disastrous results. Finding an auxiliary examination that can quickly and accurately distinguish infection from non-infection is particularly important.

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Bronchoscopy has a unique advantage in the diagnosis of PID, such as pneumonia [8]. Bronchoalveolar lavage fluid (BALF) acquired via bronchoscopy can be used for conventional microbial test (CMT) methods and metagenomic next-generation sequencing (NGS) to identify pathogens. Compared with CMTs, mNGS is more superior in efficiency [9]. In non-tumor immunocompetent patients with suspected pulmonary infection, BALF mNGS was able to effectively distinguish PNID from PID [10,11]. However, many hospitals do not have the conditions for in-house mNGS tests, they have to rely on commercial organizations for NGS testing. So far it is unclear how the sensitivity and specificity of commercial BALF mNGS change in lung cancer patients.

This study aimed to determine whether commercial mNGS of BALF could differentiate between infectious and Non-infectious pulmonary lesions in patients with lung cancer; and accurately identify pathogens responsible for the infection, therefore enhancing the accuracy of clinical diagnosis.

Methods

Study design and eligibility criteria

This study was a real-world retrospective review based on electronic medical records of lung cancer patients with bronchoalveolar lavage (BAL) and BALF commercial mNGS testing as part of clinical care from 1 April 2019 through 30 April 2022 at the First Affiliated Hospital of Sun Yat-sen University. BAL was performed using a bronchoscope produced by OLYMPUS (BF-1T260). As the standard operating procedure (SOP) of BAL in our hospital, 10–15 mL of BALF is collected for testing during the BAL process. The BALF samples were sent to commercial mNGS labs within 6 h after collection. The order of commercial mNGS testing was not interfered with by researchers of this study. No treatment decisions were made or altered as a result of this study. The First Affiliated Hospital of Sun Yat-sen University is a class A tertiary hospital in Guangzhou, China. All patients reviewed had mNGS testing in the inpatient setting. All participants signed a written informed consent form before the procedures of bronchoscope and BAL.

The study included patients who met the following criteria: (1) having any type of lung cancer; (2) showing symptoms suggestive of a pulmonary infection such as fever, cough, or difficulty breathing, along with new findings on chest CT scans; (3) undergoing bronchoscopy and BAL procedures, with BAL fluid (BALF) being collected for mNGS during their hospital stay; (4) having BALF samples available for CMTs such as culture and BALF mNGS within 48 h of admission or the onset of new symptoms; (5) having complete electronic medical records documented.

The exclusion criteria were (1) suffering from any of the following: a) primary immune deficiency diseases; b) organ transplantation; c) hematopoietic stem cell transplantation; d) lung metastasis of other tumors; e) hematological malignancy, treatment naïve or not; (2) mugs and CMTs samples were not collected at the same site of bronchi; and (3) incomplete electronic medical records.

Patient data

A total of 164 patients were included in the study, based on final diagnoses, these patients were categorized into two groups: PNID (n = 64) and PID (n = 100). Patient demographics, underlying medical conditions, CT images, and immune statuses were recorded. Commercial mNGS results were also recorded and evaluated. The data for all BALF CMTs (culture, smear, organism-specific PCR, antibody testing. Details were listed in Suppl. Table S1.) were ordered using the same batch of BALF as NGS. There are no institutional guidelines or limitations for mNGS ordering at The First Affiliated Hospital of Sun Yat-sen University.

Diagnosis of pulmonary infectious disease/pulmonary non-infectious disease

The diagnosis of pulmonary infectious and non-infectious diseases was determined by two specialized experts in the fields of pulmonary and critical care medicine. They thoroughly reviewed each patient's electronic medical records, which included clinical symptoms, laboratory tests (such as complete blood counts, bronchoalveolar lavage fluid next-generation sequencing results), CT scan images, and responses to treatment. In case of any disagreement between the two specialists, they resolved it through discussion. If a consensus could not be reached, they sought the opinion of another senior specialist.

Definitions and statistical analyses

The final diagnosis was regarded as the gold standard, and both the conventional method and BALF mNGS were examined and compared accordingly. Discordant results were defined as cases where additional organisms were detected by mNGS or when there was a discrepancy between the organisms identified by mNGS and CMTs. To ascertain the impact of these discordant mNGS results, electronic patient records were examined to determine if any antimicrobials were added or modified based on these findings. Descriptive statistical analyses were conducted on laboratory tests, including white blood cell count, neutrophils, lymphocytes, eosinophils, CRP, and PCT levels. If the data did not follow a normal distribution, the Mann-Whitney test was employed to determine the statistical significance of any observed differences. All statistical analyses were performed using GraphPad Prism software, version 9.0.

Data availability

The data used to compare the test performance of mNGS to that of CMTs, and the clinical impact of unique organisms identified by mNGS, are included in Suppl. Table S2 in the supplemental material.

Results

Demographic and clinical characteristics

A total of 164 patients with suspected pulmonary infections were included in the final analysis. There were 64 patients in the PNID group (39.0%) and 100 patients in the PID group (61%) (Table 1). The differences in age and sex were

TABLE 1

Demographic and clinical characteristics of patients enrolled

	Non-infectious disease (n = 64, 39%)	Infectious disease (n = 100, 61%)	*p*-value
Age (years)	62 (57)	62 (45)	0.7907
Male, n (%)	44 (68.75%)	78 (78%)	0.2702
Immunocompromised, n (%)	3 (4.69%)	38 (38%)	**<0.0001**
Empirical use of antibiotics, n (%)	32 (50%)	78 (78%)	**0.0002**
Laboratory findings			
White blood cell (109/L)$	7.79 (18.05)	7.53 (35.78)	0.3035
Neutrophils (109/L)$	5.28 (15.04)	5.64 (33.05)	**0.0410**
Neutrophils (%)&	69.88 ± 10.77	75.98 ± 12.38	**0.0015**
Lymphocytes (109/L)$	1.23 (11.23)	1.04 (3.21)	**0.0037**
Lymphocytes (%)&	19.62 ± 11.89	14.13 ± 8.73	**0.0008**
Eosinophils (109/L)$	0.16 (2.00)	0.11 (0.96)	**0.0123**
Eosinophils (%)&	3.47 ± 4.03	2.36 ± 2.79	**0.0393**
CRP (mg/L)$	15.76 (226.2)	36.59 (325.6)	0.0771
PCT (ng/mL)$	0.06 (1.25)	0.09 (27.92)	0.2469
CT findings of bronchoalveolar lavage site			
Bilateral pulmonary lesions, n (%)	18 (28.13%)	41 (41%)	0.0963
Consolidation, n (%)	14 (21.88%)	15 (15%)	0.2971
Solid nodules, n (%)	13 (20.31%)	16 (16%)	0.5319
Ground-glass opacities, n (%)	6 (9.38%)	11 (11%)	>0.9999
Atelectasis, n (%)	7 (10.94%)	6 (6%)	0.2742
Cavities, n (%)	3 (4.69%)	5 (5%)	>0.9999
Tree-in-bud infiltrates, n (%)	2 (3.13%)	3 (3%)	>0.9999
Bronchiectasis, n (%)	1 (1.56%)	1 (1%)	>0.9999
Unclear, n (%)	0 (0%)	1 (1%)*	>0.9999

Note: CRP, C-reactive protein; PCT, procalcitonin; CT, Computed tomography. & Mean ± standard deviation values. $ Median (interquartile range). *BALF was randomly collected because the patient had a panic attack during bronchoalveolar lavage. Statistical significance is shown in bold font.

insignificantly in the two groups. More patients were Immunocompromised (Fig. 1A 38 *vs.* 3 $p < 0.0001$) and received empiric antibiotic therapy before bronchoscopy and BAL in the PID group (Fig. 1A 78 *vs.* 32, $p = 0.0002$). As for laboratory blood test results, including white blood cell count, CRP, and PCT, showed no significant differences in the two groups, while the cell count and ratio of neutrophils, lymphocytes, and eosinophils were significantly higher in the PID group. Concerning CT findings of the BAL site, no significant differences were found in the bilateral lesions, consolidation, ground-glass opacities, solid nodules, tree-in-bud infiltrates, atelectasis, or cavities. One patient in the PID group had a panic attack during BAL; therefore, the scope could not reach the site suspected of infection, and the BALF sample was extracted from a random site in the bronchus (Fig. 1B). The PID group had less stage III (26% *vs.* 42.19%, $p = 0.0399$) and more stage I (11% *vs.* 3.13%, $p < 0.001$) patients compared to PNID group (Fig. 1C).

Distributions of pulmonary infectious pathogens and non-infectious diseases.

There was no significant difference in the pathological type of lung cancer (Fig. 1D & Table 2).

According to Table 3, among the 52 patients with PID, bacterial infections were the most common cause of infection. *Staphylococcus aureus* was detected in 8% of patients, making it the most commonly found pathogen. This was followed by *Klebsiella pneumoniae*, Nontuberculosis mycobacteria, *Pseudomonas aeruginosa*, and *Streptococcus constellation*, each accounting for 5% of the cases (as shown in Fig. 2A). Fungal infections were observed in 32% of patients, with the most common types being *Pneumocystis Yersini* (17%) and *Aspergillus* (6%). Additionally, one patient was found to be infected with *Talaromyces marneffei* (Fig. 2B).

Comparison of diagnostic performance for differentiating PID from PNID with bronchoalveolar lavage fluid mNGS or conventional microbial tests

Fig. 3A displays the positivity rates of BALF mNGS and CMTs for PID and PNID. The positive predictive value for diagnosing PID using BALF mNGS was 83.5%, with a

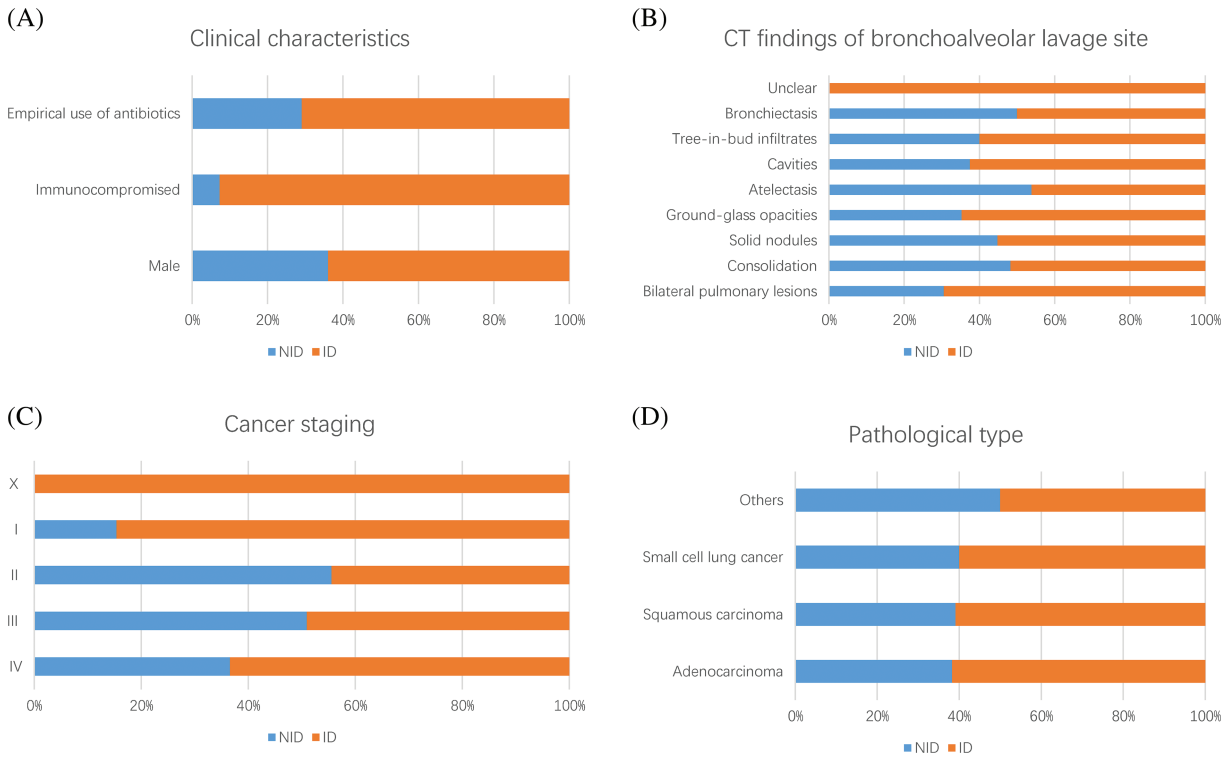

FIGURE 1. The ratio of clinical and cancer-related characteristics of patients enrolled. (A) Clinical characteristics of NID and ID groups. ID group is more likely to be immune-compromised (ID *vs.* NID = 38% *vs.* 4.69%, $p < 0.0001$) and empirically given antibiotics (ID *vs.* NID = 78% *vs.* 50%, $p = 0.0002$). (B) CT findings of bronchoalveolar lavage site of NID and ID groups. (C) Cancer staging of NID and ID groups. ID group has more stage I patients (ID *vs.* NID = 11% *vs.* 3.13%, $p < 0.0001$). (D) Pathological type of NID and ID groups. CT: computed tomography, NID: non-infectious disease, ID: infectious disease.

negative predictive value of 71.6%. The likelihood ratio was 3.24. Comparing BALF mNGS to CMTs, it was observed that BALF mNGS increased the sensitivity rate by approximately 60% (81% *vs.* 21%, $p < 0.05$), while there was no significant difference in specificity (75% *vs.* 98.4%,

TABLE 2

Cancer-related characteristics

	Non-infectious disease (n = 64)	Infectious disease (n = 100)	*p*-value
Pathological type			
Adenocarcinoma, n (%)	44 (68.75%)	71 (71%)	0.8614
Squamous carcinoma, n (%)	9 (14.06%)	14 (14%)	>0.9999
Small cell lung cancer, n (%)	8 (12.50%)	12 (12%)	>0.9999
Others, n (%)	3 (4.69%)	3 (3%)	0.6791
Staging			
IV (%)	30 (46.88%)	52 (52%)	0.6312
III (%)	27 (42.19%)	26 (26%)	**0.0399**
II (%)	5 (7.81%)	4 (4%)	0.4845
I (%)	2 (3.13%)	11 (11%)	**<0.0001**
X (%)	0 (0%)	7 (7%)	0.0434

Note: Statistical significance is shown in bold font.

TABLE 3

Pathogens found in infectious diseases

Pathogen	n (%)*	%
Bacteria	52	52
Staphylococcus aureus	8	8
Klebsiella pneumoniae	5	5
Nontuberculosis mycobacteria	5	5
Pseudomonas aeruginosa	5	5
Streptococcus constellation	5	5
Haemophilus parainfluenza	4	4
Mycobacterium tuberculosis	4	4
Stenotrophomonas maltophilia	4	4
Haemophilus influenza	3	3
Acinetobacter baumannii	3	3
Enterococcus faecium	2	2
Others#	4	4
Fungi	32	32
Pneumocystis Yersini	17	17
Aspergillus spp.	6	6
Candida spp.	4	4
Pneumocystis carinii	4	4
Talaromyces marneffei	1	1

Note: *Number of patients, with the percentage in parentheses. #Including *Escherichia coli* (n = 1); *Moraxella catarrhalis* (n = 1); *Parvimonas Micra* (n = 1); *Prevotella melaninogenica* (n = 1).

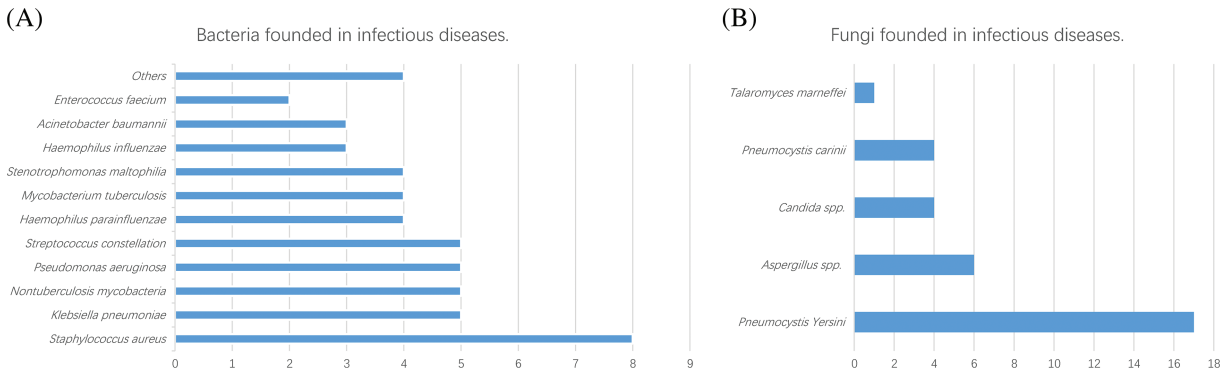

FIGURE 2. Pathogens found in infectious diseases. (A) Bacteria detected via BALF my test. *Staphylococcus aureus* (8%) was the most commonly detected pathogen, followed by *Klebsiella pneumoniae* (5%), nontuberculosis mycobacteria (5%), *Pseudomonas aeruginosa* (5%) and *Streptococcus constellation* (5%). (B) Fungi were found in the ID group. The most commonly detected fungi were *Pneumocystis yersini* (17%) and *Aspergillus* (6%). Aspergillus spp.: Aspergillus species, Candida spp.: Candida species. BALF: bronchoalveolar lavage fluid, ID: infectious disease, mNGS: metagenomic next-generation sequencing.

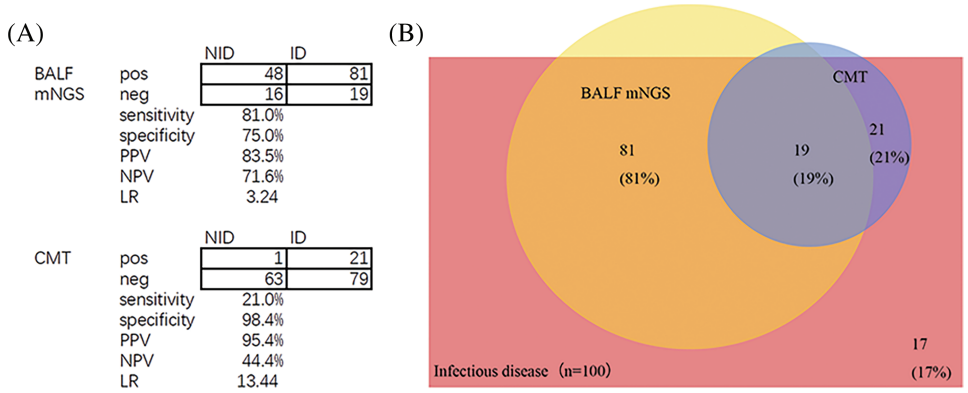

FIGURE 3. Sensitivity and specificity of mNGS and CMTs. (A) Sensitivity and specificity of mNGS and CMTs. The BALF mNGS demonstrated a positive predictive value of 83.5% and a negative predictive value of 71.6% for diagnosing infectious diseases. Compared to CMTs, BALF mNGS increased the sensitivity rate by approximately 60% (81% *vs.* 21%; $p < 0.01$). (B) Moreover, despite comprehensive mNGS yielding negative results, two patients (2%) still tested positive for BALF CMT, indicating a non-infectious disease (NID) rather than an infectious disease (ID) scenario. mNGS: metagenomic next-generation sequencing, PPV: positive predictive value, NPV: negative predictive value, LR: likelihood ratio.

$p > 0.1$) (Fig. 1A). Out of the 100 patients, only two were confirmed by CMT, resulting in a low positive rate of 21% for CMT, whereas the positive rate for mNGS was 81% (Fig. 3B). For additional details, please refer to Suppl. Table S2.

Diagnosis assisted by bronchoalveolar lavage fluid mNGS for pneumocystis

Among the 100 patients diagnosed with PID, 17 were confirmed *Pneumocystis yersini* infection by BALF mNGS, while one was confirmed by CMTs (Suppl. Table S2). Notably, all four cases of *Pneumocystis carinii* infection were BALF mNGS-positive despite comprehensive CMTs being negative, indicating mNGS is more sensitive in detecting Pneumocystis.

Immune status affects patients

As demonstrated in Fig. 4, PNID and PID made no significant difference in affecting OS of lung cancer patients (Fig. 4A, median OS NID *vs.* ID = 44 *vs.* 60 (months), $p = 0.8904$). On the other hand, patients with defective immunity had shorter OS than immunology intact patients (Fig. 4B median OS immunology defect *vs.* immunology intact = 60 *vs.* 77 (months), $p = 0.0285$). One main reason for this phenomenon is that patients with defection in immunity came with a heavier burden of tumor load and went through more cycles of treatments, impacting their immunological status.

Tyrosine kinase inhibitors (TKIs) cause longer overall survival in lung cancer patients

Patients with TKI therapy possess longer OS than other anti-cancer agents, except for immune checkpoint inhibitors (ICIs); the difference between TKIs and ICIs was insignificant (Fig. 5B, median OS TKIs *vs.* ICIs *vs.* anti-angiogenic *vs.* chemo *vs.* radiotherapy = 76 *vs.* 84 *vs.* 61 *vs.* 58 *vs.* 60 (months), p (TKIs *vs.* ICIs) = 0.0570, p (TKIs *vs.* anti-angiogenic) = 0.0297, p (TKIs *vs.* chemo) = 0.0028, p (TKIs *vs.* radiotherapy) = 0.0064). The differences among ICIs, anti-angiogenic, chemo-, and radiotherapy were insignificant. Moreover, the pathological of lung cancer made no significant differences to OS (Fig. 5A, $p = 0.1023$). However, when comparing the OS caused by TKI treatment

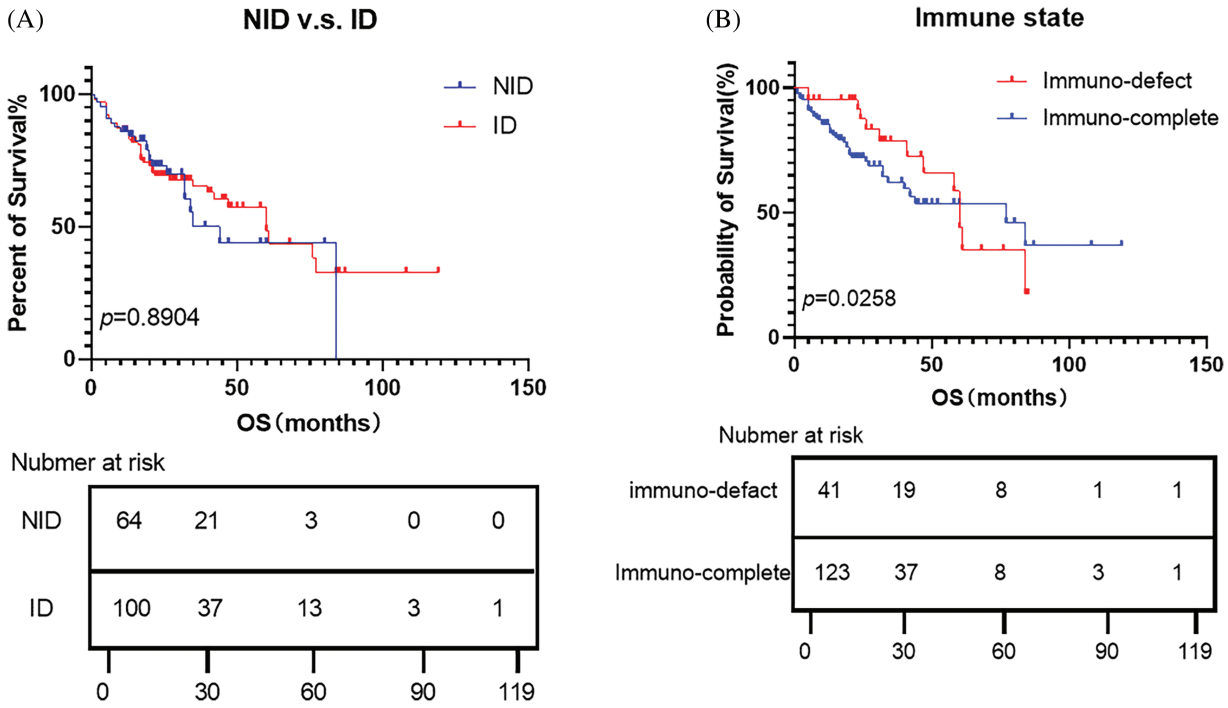

FIGURE 4. OS of non-infectious disease, infectious disease, and different immune states. (A) The OS between NID and ID groups did not differ significantly (p = 0.8904). (B) The immuno-complete group possesses better OS compared to Immuno-defect (p = 0.0258). NID: non-infectious disease, ID: infectious disease, OS: overall survival.

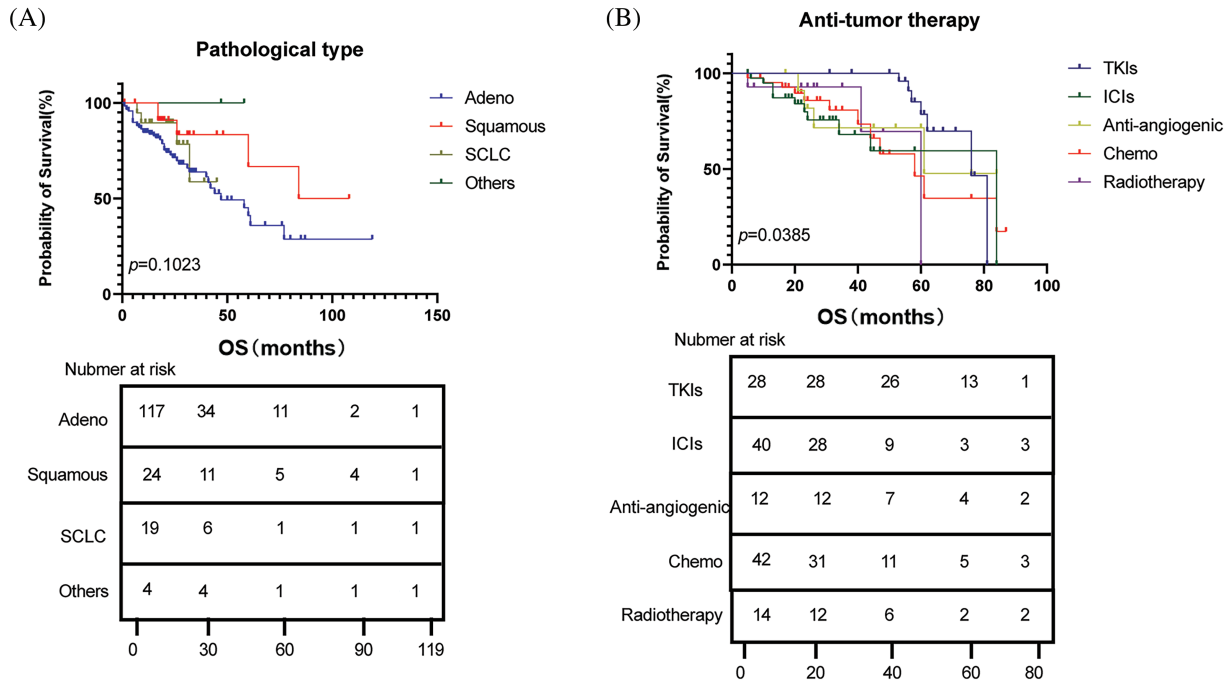

FIGURE 5. OS of different cancer pathological types and anti-tumor therapy. (A) Pathological type does not affect patients' OS (p = 0.1023). (B) TKI therapy entails longer OS than other anti-cancer agents, median OS TKIs *vs.* ICIs *vs.* Anti-angiogenic *vs.* Chemo *vs.* Radiotherapy = 76 *vs.* 84 *vs.* 61 *vs.* 58 *vs.* 60 (months), p (TKIs *vs.* ICIs) = 0.0570, p (TKIs *vs.* anti-angiogenic) = 0.0297, p (TKIs *vs.* Chemo) = 0.0028, p (TKIs *vs.* radiotherapy) = 0.0064. Adeno: adenocarcinoma, Squamous: squamous carcinoma, SCLC: small cell lung cancer, TKIs: tyrosine kinase inhibitors, ICIs: immuno-checkpoint inhibitors, Anti-angiogenic: antiangiogenic agents, Chemo: chemotherapy, OS: overall survival.

brought in either PNID or PID patients, the difference was insignificant (Fig. 6A, median OS PNID *vs.* PID=NE *vs.* 77 (months), p = 0.1971). OS did not differ between ID and NID groups, irrespective of the treatment patients were given (Figs. 6B–6E).

Discussion

Our study showed that in patients with lung cancer, compared with CMTs, commercial mNGS using BALF had 60% higher diagnostic sensitivity and effectively filled the gap when

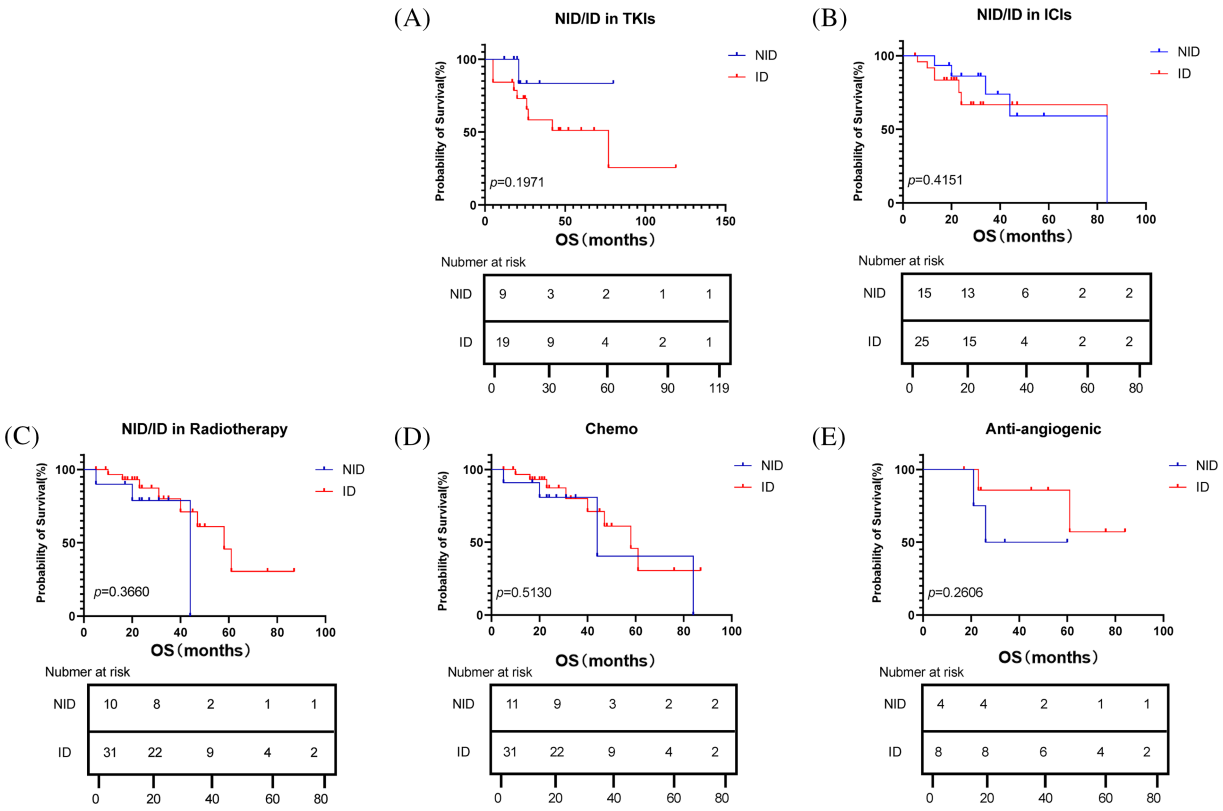

FIGURE 6. OS of different anti-tumor therapy in NID or ID patients. (A–E) No difference in OS was observed between ID and NID groups, regardless of anti-cancer treatments. TKIs: tyrosine kinase inhibitors, ICIs: immuno-checkpoint inhibitors, anti-angiogenic: antiangiogenic agents, Chemo: chemotherapy, NID: non-infectious disease, ID: infectious disease.

CMT evidence was not sufficient, regardless of pathological types.

Infection and tumors are interrelated and influence each other on many levels [12–14]. Infection and cancer can both lead to continuous stimulation of T-cells, and exhausted T-cells gradually lose effector function and characteristics of memory T-cells [15,16]. Patients with lung cancer are at greater risk of dying from pneumonia [17]; therefore, accurate and quick identification of pathogens is the key to reducing mortality and prolonging the OS of lung cancer patients with ID. Regardless of the underlying mechanism, most of the pathogens negatively affect OS and the quality of life of patients with lung cancer, with or without anti-cancer treatments [18,19]. What's more, antibiotic administration was significantly associated with worsened PFS in lung cancer patients treated with ICIs [12–14]; therefore, it is also important to reduce the unnecessary prescription of antibiotics. As anti-cancer therapies are being developed, PNID caused by ICIs and TKIs brings challenges to the management of lung cancer [20–22]. It urgently needs a reliable method to distinguish the nature of new lesions found in CT imaging of patients with lung cancer.

Generally speaking, BAL is a safe method for patients with possible lung diseases. The common complications of BAL are bleeding, fever, laryngeal edema, and hypoxia. With adequate preoperative preparation, most of the complications are preventable. In addition to collecting BALF for testing, the process of BAL can also flush the patient's accumulated sputum in the bronchus, providing an unobstructed pulmonary drainage pathway. As an important means of diagnosis and treatment of lung diseases, bronchoscopes, and BAL have been widely used in clinical work.

CMTs, or complete blood counts, are widely utilized in medical diagnostics and can provide insight into the degree of infection [23]. Physicians in both primary care settings and specialized hospitals typically rely on routine blood tests to make accurate infection diagnoses [24]. To identify ID, cell count and the ratio of neutrophils was proved reliable in our study. However, in contrast to the present study and clinical practice experience, Zhu's study found no difference in blood routine indexes between PNID and PID groups [25]. Therefore, blood routine cannot be used alone to diagnose PID. Moreover, routine blood tests cannot determine the type of pathogen. The liquid-based culture method is capable of sorting out the pathogen in blood, BALF, or other body fluids [26] but the limited number of pathogens could be detected at the same time causing a repetition of the tests [27].

Compared to CMTs, mNGS is broad-spectrum, and has a faster detection process than the liquid culture method, with a higher positive rate in a single test [28,29]. Although the specimens to be tested by NGS can be different kinds of body fluids, blood is still the most commonly used [30]. According to Sun's study, BALF mNGS is more sensitive and exhibits better diagnostic accuracy in patients with suspected severe pneumonia [31], indicating that BALF is

more suitable for identifying PID/PNID in patients with lung cancer.

As a new method of auxiliary examination, mNGS is expensive, and unaudited usage increases the financial burden of patients [32,33]. Primary hospitals usually cannot afford in-house mNGS stations; therefore, the accessibility of commercial mNGS tests could better help patients in primary hospitals when facing PID. In our study, compared to CMTs, commercial mNGS showed great potential in diagnosing PID, especially when smear or staining in CMTs cannot offer sufficient evidence.

However, certain limitations of this study must be stressed. First, the NGS tests evaluated in this study were offered by commercial test centers, unlike CMTs, which were carried out in the in-house laboratory. The transportation from the hospital to the commercial lab may affect the accuracy of the NGS results even though an SOP of the cold chain was applied. Second, the present study was single-center research, and the randomness of cases involved in the study was limited due to the nature of real-world retrospective studies. Further randomized control trial is needed to rule out the physician`s subjective influence in BAL implementation.

Conclusions

In conclusion, our study indicates that BALF mNGS can add value by improving overall sensitivity in lung cancer patients with suspected pulmonary infection and was outstanding in identifying *Pneumocystis* infection. It may help physicians to determine the follow-up treatment adjustment and avoid the abuse of antibiotics. In primary hospitals without access to complex equipment and in-house mNGS testing instruments, commercial BALF mNGS could be a safe, convenient, and economical method that can improve the accuracy of PNID and PID diagnoses in lung cancer patients. In identifying pathogens in patients with intact immune function, BALF mNGS is superior to the combination of CMTS, the combination of mNGS and CMTs may be a better diagnostic strategy.

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Author Contributions: Study conception and design: Kejing Tang and Jiyu Wang; data collection: Deyuan Zhou and Yuxia Li; analysis and interpretation of results: Jiyu Wang, Lihong Bai, Kejing Tang; draft manuscript preparation: Jiyu Wang and Lihong Bai. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval: The research plan and electronic medical records of patients involved in this study were reviewed and approved by the Ethics Committee of the First Affiliated Hospital, Sun Yat-sen University. ethical approval code: IIT-2023-145. Date of approval: 2nd March 2023. This study does not interfere with clinical decisions.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Materials

TABLE S1

Conventional microbiological tests methods in the study

Pathogen	Technique	Sample	Microbiological methods/tests
Bacterial	Bacterial culture	BALF	Bacterial culture on appropriate media with bacterial identification by MALDI-TOF mass spectrometry
	Smear	BALF	Acid-fast staining for *Mycobacteria*
Fungal	Fungal culture	BALF	Fungal culture on appropriate media with fungal identification by MALDI-TOF mass spectrometry
	Smear	BALF	India ink staining for *Cryptococcus*; hexaamine silver staining for *Pneumocystis*
	Antigen detection	BALF	Cryptococcal antigen and galactomannan antigen
Viral	PCR	BALF	Influenza A/B, Epstein-Barr virus, Human herpesvirus, and Cytomegalovirus

Note: BALF, bronchoalveolar lavage fluid; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PCR, polymerase chain reaction.

TABLE S2

Sample details of enrolled cases

Case number	Gender (M/F)	Age	Immuno-defect ?	Final clinical diagnosis	Conventional microbiological tests result	BALF mNGS result	Final confirmed pathogens
1	M	55	N	ID	Negative	*Staphylococcus aureus*; *Mycobacterium tuberculosis*;	*Staphylococcus aureus*
2	M	71	Y	ID	Negative	*Candida albicans*	*Candida albicans*
3	M	58	Y	ID	Negative	*Aspergillus fumigatus*	*Aspergillus fumigatus*
4	M	47	Y	ID	Pneumocystis Yersini	Pneumocystis Yersini	Pneumocystis Yersini
5	M	63	N	NID	Negative	Negative	None
6	M	55	N	ID	*Mycobacterium tuberculosis*	*Mycobacterium tuberculosis*	*Mycobacterium tuberculosis*
7	M	53	N	ID	Parainfluenza virus; Cytomegalovirus; Epstein-Barr virus	*Streptococcus pneumoniae*; Pneumocystis Yersini; Cytomegalovirus; Epstein-Barr virus	Pneumocystis Yersini
8	F	55	N	ID	*Stenotrophomonas maltophilia*; *Candida albicans*	Corynebacterium propionate; Virococcus viridis	Uncertain
9	F	55	N	ID	Negative	Corynebacterium propionate; Virococcus viridis	Uncertain
10	M	65	N	ID	Negative	Capnocytophaga	Uncertain
11	M	67	Y	NID	Epstein-Barr virus	Epstein-Barr virus	None
12	F	67	N	ID	*Candida albicans*	*Streptococcus intermedius*; Streptococcus constellation; Streptomyces vulgaris	Streptococcus constellation
13	M	53	N	NID	Negative	Negative	None
14	M	59	N	ID	Negative	Prevotellaceae melaninogenica; Prevotellaceae pallens	Uncertain
15	F	62	N	NID	Negative	Negative	None
16	M	49	Y	ID	Negative	*Haemophilus parainfluenzae*; *Streptococcus pneumoniae*	*Haemophilus parainfluenzae*; *Streptococcus pneumoniae*
17	M	64	Y	ID	Negative	Negative	Uncertain
18	M	67	N	ID	Negative	*Acinetobacter baumannii*; *Streptococcus pneumoniae*; Coliform bacteria	*Streptococcus pneumoniae*
19	M	61	N	NID	Negative	Negative	None
20	M	68	Y	ID	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
21	M	76	Y	ID	Negative	*Pneumocystis carinii*	*Pneumocystis carinii*
22	M	59	N	ID	Negative	Negative	Uncertain
23	M	71	N	NID	Negative	Negative	None
24	M	62	Y	ID	Negative	*Haemophilus influenzae*	*Haemophilus influenzae*
25	M	56	N	ID	Negative	*Haemophilus parainfluenzae*; Coliform bacteria	*Haemophilus parainfluenzae*
26	M	68	Y	ID	Negative	*Streptococcus pneumoniae*; *Klebsiella pneumoniae*	*Klebsiella pneumoniae*
27	M	60	Y	ID	*Aspergillus flavus*	Negative	*Aspergillus flavus*
28	M	69	N	NID	Negative	*Streptococcus pneumoniae*	None

(Continued)

Table S2 (continued)

Case number	Gender (M/F)	Age	Immuno-defect ?	Final clinical diagnosis	Conventional microbiological tests result	BALF mNGS result	Final confirmed pathogens
29	F	71	N	NID	Negative	Negative	None
30	M	57	N	ID	*Stenotrophomonas maltophilia*	*Enterococcus faecium*; *Stenotrophomonas maltophilia*	*Enterococcus faecium*
31	M	57	Y	ID	Negative	Pneumocystis Yersini	Pneumocystis Yersini
32	M	60	N	ID	Negative	Negative	Uncertain
33	M	69	N	ID	*Candida albicans*	Pneumocystis Yersini; *Candida albicans*	Pneumocystis Yersini
34	M	64	N	NID	Negative	*Haemophilus parahaemolyticus*	None
35	M	63	Y	NID	Negative	Negative	None
36	M	68	N	ID	Negative	*Haemophilus parainfluenzae*	*Haemophilus parainfluenzae*
37	F	49	N	ID	Negative	*Isoptericola variabilis*	Uncertain
38	F	57	N	NID	Negative	Negative	None
39	M	76	N	NID	Negative	Negative	None
40	F	64	N	ID	Negative	Parvimonas Micra	Parvimonas Micra
41	M	64	N	ID	Nontuberculosis mycobacteria	Nontuberculosis mycobacteria	Nontuberculosis mycobacteria
42	F	62	N	NID	Negative	*Streptococcus pneumoniae*	None
43	M	69	N	NID	Negative	Negative	None
44	M	65	N	NID	Negative	Negative	None
45	M	69	N	ID	*Pseudomonas aeruginosa*; *Candida albicans*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
46	M	63	Y	ID	Negative	Moraxella catarrhalis	Moraxella catarrhalis
47	M	60	N	ID	Negative	Negative	Uncertain
48	M	43	Y	ID	Negative	*Staphylococcus aureus*	*Staphylococcus aureus*
49	F	56	N	NID	Negative	Negative	None
50	F	50	N	NID	Negative	Negative	None
51	M	65	N	ID	Negative	Negative	Uncertain
52	M	63	N	NID	Negative	Negative	None
53	M	38	N	ID	Negative	*Haemophilus influenzae*; *Acinetobacter baumannii*; *Streptococcus pneumoniae*; Streptococcus parahaemolyticus	Uncertain
54	M	64	N	ID	*Staphylococcus aureus*; *Candida albicans*	*Staphylococcus aureus*; *Candida albicans*	*Staphylococcus aureus*
55	M	65	N	ID	*Mycobacterium tuberculosis*	*Mycobacterium tuberculosis*; Epstein-Barr virus	*Mycobacterium tuberculosis*
56	F	56	Y	ID	*Candida albicans*	Pneumocystis Yersini	Pneumocystis Yersini
57	M	72	N	ID	Negative	*Klebsiella pneumoniae*; *Streptococcus pneumoniae*; *Mycoplasma hominis*; Epstein-Barr virus; HPV	*Klebsiella pneumoniae*
58	M	69	N	NID	Negative	Negative	None
59	M	59	N	ID	Negative	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*

(Continued)

Table S2 (continued)

Case number	Gender (M/F)	Age	Immuno-defect ?	Final clinical diagnosis	Conventional microbiological tests result	BALF mNGS result	Final confirmed pathogens
60	F	58	N	NID	Negative	*Klebsiella pneumoniae*	None
61	M	33	N	NID	Negative	Negative	None
62	M	43	Y	ID	Negative	*Staphylococcus aureus*	*Staphylococcus aureus*
63	M	76	N	NID	Negative	Negative	None
64	M	54	N	ID	*Candida albicans*; *Candida parapsilosis*	*Pseudomonas aeruginosa*; *Klebsiella pneumoniae*; Saccharomyces cerevisia	*Pseudomonas aeruginosa*
65	F	51	N	NID	Negative	Negative	None
66	F	57	Y	ID	Negative	*Staphylococcus aureus*; *Haemophilus parahaemolyticus*; *Streptococcus pneumoniae*; *Klebsiella pneumoniae*; *Enterococcus faecium*	*Staphylococcus aureus*
67	F	57	Y	ID	*Candida albicans*	*Enterococcus faecalis*; *Candida albicans*; *Haemophilus influenzae*	*Candida albicans*
68	M	59	N	ID	Negative	Negative	Uncertain
69	M	59	N	NID	Negative	Negative	None
70	M	57	N	NID	Negative	Negative	None
71	M	61	N	NID	Negative	Negative	None
72	F	62	N	NID	Negative	*Staphylococcus aureus*	None
73	M	60	N	ID	Negative	*Haemophilus parainfluenzae*	*Haemophilus parainfluenzae*
74	M	68	N	ID	Negative	*Klebsiella pneumoniae*; Epstein-Barr virus	*Klebsiella pneumoniae*
75	M	60	N	NID	Negative	Negative	None
76	M	65	Y	ID	Negative	Negative	Uncertain
77	M	55	N	NID	Negative	*Stenotrophomonas maltophilia*; Streptococcus parasanguis	None
78	M	71	N	ID	*Candida albicans*	*Prevotella melaninogenica*	*Prevotella melaninogenica*
79	M	67	N	NID	Negative	Negative	None
80	F	50	N	NID	Negative	Negative	None
81	M	55	N	NID	Negative	Negative	None
82	M	69	N	NID	Negative	*Prevotella melaninogenica*	None
83	F	53	N	ID	Negative	*Staphylococcus aureus*; *Haemophilus influenzae*	*Staphylococcus aureus*
84	M	58	N	ID	Negative	Negative	Uncertain
85	F	51	N	NID	Negative	Negative	None
86	M	71	N	ID	Negative	Pneumocystis Yersini	Pneumocystis Yersini
87	F	62	N	ID	Negative	Pneumocystis Yersini	Pneumocystis Yersini
88	M	76	N	ID	*Aspergillus fumigatus*	Negative	*Aspergillus fumigatus*
89	M	66	Y	ID	Negative	Pneumocystis Yersini	Pneumocystis Yersini
90	M	58	N	NID	Negative	Negative	None
91	M	61	N	NID	Negative	Negative	None
92	M	64	N	NID	Negative	Negative	None

(Continued)

Table S2 (continued)

Case number	Gender (M/F)	Age	Immuno-defect ?	Final clinical diagnosis	Conventional microbiological tests result	BALF mNGS result	Final confirmed pathogens
93	M	69	Y	ID	Negative	*Acinetobacter baumannii*; *Klebsiella pneumoniae*; *Candida albicans*; Human herpesvirus 1	*Acinetobacter baumannii*
94	M	63	N	NID	Negative	Negative	None
95	F	60	Y	ID	Negative	Negative	Uncertain
96	F	69	N	NID	Negative	*Staphylococcus aureus*	None
97	M	75	Y	ID	Cytomegalovirus	*Pneumocystis carinii*; Cytomegalovirus	*Pneumocystis carinii*
98	F	31	N	NID	Negative	Negative	None
99	M	53	N	NID	Negative	Rhinovirus	None
100	M	53	Y	NID	Negative	Negative	None
101	M	69	Y	ID	Negative	*Pneumocystis carinii*; Streptococcus hemolyticus; Human herpesvirus 4	*Pneumocystis carinii*
102	M	62	N	NID	Negative	Negative	None
103	M	68	N	ID	Negative	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
104	F	58	Y	ID	Negative	Pneumocystis Yersini	Pneumocystis Yersini
105	F	57	N	NID	Negative	*Haemophilus influenzae*; *Aspergillus fumigatus*	None
106	F	54	N	NID	Negative	Negtive	None
107	F	31	N	ID	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
108	M	44	Y	ID	Coliform bacteria	*Staphylococcus aureus*; Coliform bacteria; Streptococcus constellation	*Escherichia coli*
109	M	58	N	NID	Negative	Pneumocystis Yersini; Epstein-Barr virus	None
110	M	70	N	ID	Negative	Nontuberculosis mycobacteria; Epstein-Barr virus	Nontuberculosis mycobacteria
111	M	68	N	ID	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
112	M	69	Y	ID	Negative	Negative	Uncertain
113	M	64	Y	ID	Negative	Pneumocystis Yersini	Pneumocystis Yersini
114	M	69	N	NID	Negative	Negative	None
115	M	68	N	ID	Negative	*Stenotrophomonas maltophilia*	Uncertain
116	M	64	Y	ID	Negative	Pneumocystis Yersini; Epstein-Barr virus; *Haemophilus parainfluenzae*	Pneumocystis Yersini
117	M	56	N	ID	Nontuberculosis mycobacteria	Nontuberculosis mycobacteria; *Pseudomonas aeruginosa*; *Legionella pneumophila*	Nontuberculosis mycobacteria
118	M	50	Y	ID	*Candida albicans*	*Stenotrophomonas maltophilia*; *Candida albicans*	*Candida albicans*
119	M	50	Y	ID	Negative	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*
120	M	72	N	NID	Negative	Negative	None
121	M	88	N	ID	Negative	*Aspergillus terreus*	*Aspergillus terreus*
122	M	48	Y	ID	Negative	Pneumocystis Yersini; *Aspergillus flavus*; Epstein-Barr virus	Pneumocystis Yersini

(Continued)

Table S2 (continued)

Case number	Gender (M/F)	Age	Immuno-defect ?	Final clinical diagnosis	Conventional microbiological tests result	BALF mNGS result	Final confirmed pathogens
123	M	63	Y	ID	*Klebsiella pneumoniae*; *Candida tropicalis*	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
124	M	63	N	ID	Negative	*Staphylococcus aureus*; *Haemophilus parainfluenzae*	*Staphylococcus aureus*
125	M	54	N	ID	Negative	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
126	M	60	N	NID	Negative	Negative	None
127	M	67	N	NID	Negative	*Candida albicans*	None
128	M	57	N	NID	Negative	rhinovirus	None
129	M	62	N	ID	Negative	*Haemophilus parainfluenzae*; *Mycobacterium tuberculosis*; Epstein-Barr virus	*Mycobacterium tuberculosis*
130	M	63	N	ID	*Aspergillus fumigatus*	*Aspergillus fumigatus*	*Aspergillus fumigatus*
131	F	58	N	ID	Negative	*Staphylococcus aureus*	*Staphylococcus aureus*
132	M	81	Y	ID	*Stenotrophomonas maltophilia*	*Aspergillus fumigatus*	*Aspergillus fumigatus*
133	M	69	N	ID	Negative	*Acinetobacter baumannii*	*Acinetobacter baumannii*
134	M	71	N	ID	*Candida albicans*	Streptococcus constellation;	Streptococcus constellation
135	M	77	N	ID	*Candida albicans*	Pneumocystis Yersini; *Candida albicans*; *Candida tropicalis*	*Candida albicans*
136	M	52	Y	ID	Negative	*Enterococcus faecium*; *Enterococcus faecalis*; Epstein-Barr virus	*Enterococcus faecium*
137	F	58	N	ID	*Aspergillus fumigatus*	*Streptococcus pneumoniae*; Pneumocystis Yersini	Pneumocystis Yersini
138	M	69	N	NID	Negative	*Penicillium digitatum*	None
139	F	79	N	ID	*Candida albicans*	*Klebsiella pneumoniae*; Pneumocystis Yersini	Pneumocystis Yersini
140	F	66	N	NID	Negative	Negative	None
141	F	69	N	ID	*Klebsiella pneumoniae*	*Mycobacterium tuberculosis*; *Klebsiella pneumoniae*	*Mycobacterium tuberculosis*; *Klebsiella pneumoniae*
142	F	64	N	ID	Negative	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*
143	M	65	N	ID	Negative	Pneumocystis Yersini; Epstein-Barr virus	Pneumocystis Yersini
144	M	71	Y	ID	*Candida albicans*	*Haemophilus influenzae*	*Haemophilus influenzae*
145	F	63	N	NID	Negative	Negative	None
146	F	57	N	ID	*Candida albicans*	Nontuberculosis mycobacteria	Nontuberculosis mycobacteria
147	F	59	N	ID	Negative	*Talaromyces marneffei*	*Talaromyces marneffei*
148	M	59	N	NID	Negative	Negative	None
149	M	65	N	NID	Negative	Rhinovirus	None

(Continued)

Table S2 (continued)

Case number	Gender (M/F)	Age	Immuno-defect ?	Final clinical diagnosis	Conventional microbiological tests result	BALF mNGS result	Final confirmed pathogens
150	F	51	Y	ID	Negative	*Pneumocystis carinii*	*Pneumocystis carinii*
151	M	58	N	ID	Negative	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*
152	M	72	Y	ID	*Candida tropicalis*	*Acinetobacter baumannii*	*Acinetobacter baumannii*
153	F	60	N	NID	Negative	Negative	None
154	M	64	N	NID	Negative	Negative	None
155	M	56	N	NID	Negative	Negative	None
156	M	63	N	NID	Negative	Negative	None
157	M	55	N	ID	Negative	Pneumocystis Yersini	Pneumocystis Yersini
158	M	70	N	NID	Negative	Negative	None
159	M	61	N	NID	Negative	Negative	None
160	F	73	N	NID	Negative	Negative	None
161	M	44	N	ID	Negative	*Staphylococcus aureus*; *Haemophilus influenzae*	*Haemophilus influenzae*
162	F	59	N	NID	Negative	Negative	None
163	M	72	N	ID	Negative	Nontuberculosis mycobacteria	Nontuberculosis mycobacteria
164	F	62	Y	ID	Negative	Pneumocystis Yersini; Light streptococcus	Pneumocystis Yersini