Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Analysis of Clinical Features in Pulmonary Infection Based on Two Next-Generation Sequencing Tests of BronchoalveolarLavage Fluid

Junzhang Qin
No. 924 Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army
Xiandong Yin
No. 924 Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army
Zhen Xiong
xiongzhen007@163.com

No. 924 Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army

Research Article

Keywords: BALF NGS (Bronchoalveolar Lavage Fluid Next-Generation Sequencing), Pulmonary Infection, Second Testing, Pathogen Spectrum, Uncommon Pathogens, Fiberoptic Bronchoscopy

Posted Date: July 1st, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9929154/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Objective: To investigate the clinical features of patients with severe pneumonia or complicated pulmonary infections who underwent two consecutive next-generation sequencing (NGS) tests of bronchoalveolar lavage fluid (BALF), and to explore changes in pathogen distribution between the two tests. The study aims to provide evidence for precise antimicrobial therapy and efficacy assessment in patients with disease progression or Uncommon Pathogens, thereby avoiding unnecessary repeat BALF NGS testing.

Methods: A total of 34 patients with severe pneumonia or complicated pulmonary infections who underwent two BALF NGS tests at the 924th Hospital of Joint Logistics Support Force of the Chinese People's Liberation Army were retrospectively enrolled. Clinical characteristics were summarized, and differences in pathogen distribution and changes in normalized sequence counts between the first and second NGS tests were analyzed in relation to disease progression.

Results: Among the 34 patients, 76.51% were male and 61.8% were over 60 years old. The most common symptoms were cough (82.41%), sputum production (58.82%), and dyspnea or wheezing (41.20%). Comorbidities were present in 79.42% of patients. Comparison between severe (n = 24) and non-severe (n = 10) groups showed that the severe group had a significantly higher prevalence of comorbidities (91.71% vs. 50%, P = 0.01) and was more frequently infected with fungi and Uncommon Pathogens, while non-severe cases were mainly associated with non-tuberculous mycobacteria (NTM) (P = 0.02). In 18 patients (53%) with disease progression, new pathogens were detected in the second NGS test in 78% of cases, including *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Five previously undetected pathogens were identified, such as *Mycobacterium tuberculosis* and *Cryptococcus*. In the follow-up group (n = 11, 32%), normalized sequence counts of pathogens such as *Aspergillus spp.*, *Talaromyces marneffei*, and *Chlamydia psittaci* significantly decreased or became undetectable, suggesting potential treatment efficacy.

Conclusion: In the second BALF NGS test, the detection counts of *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Staphylococcus haemolyticus* increased significantly. Pathogens in the severe group were predominantly fungi and atypical microorganisms. In the clinical deterioration group, the main causative pathogens of new infections were *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. In contrast, infections with non-tuberculous mycobacteria (NTM), fungi, and atypical pathogens served as the primary indications for undergoing a second BALF NGS test in the routine follow-up group. These findings provide important reference for identifying potential pathogens and guiding anti-infective therapy when clinical deterioration occurs in patients with severe pneumonia or complicated pulmonary infections, thereby avoiding unnecessary repeat BALF NGS testing and reducing the financial burden on patients. More critically, in the routine follow-up group, the normalized sequence counts of certain pathogens decreased significantly or even became undetectable compared with the first test. This trend may serve as an objective molecular

biomarker for evaluating treatment efficacy against specific pathogens such as non-tuberculous mycobacteria, suggesting potential clinical application value.

Introduction

Pulmonary infections, particularly community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP), remain among the leading causes of morbidity and mortality worldwide[1]. In recent years, changes in pathogen distribution have been observed, largely due to the widespread and often inappropriate use of broad-spectrum antibiotics, as well as the increasing application of immunosuppressants and corticosteroids[2]. Gram-negative bacteria, respiratory viruses, and multidrug-resistant organisms are now more frequently identified as causative agents. Severe pneumonia and chronic pulmonary infections represent more serious clinical manifestations, often occurring in patients with multiple comorbidities and prolonged treatment courses.

In such patients, clinical deterioration during therapy or infection with Uncommon Pathogens not detectable by conventional methods may indicate superimposed infections or treatment failure. Understanding the distribution of causative pathogens in these complex cases is essential for guiding clinical decisions. Conventional diagnostic methods, including smears, cultures, and immunoassays, often have limited sensitivity, especially after initial targeted antimicrobial treatment based on first-round bronchoalveolar lavage fluid metagenomic next-generation sequencing (BALF NGS). These traditional methods are particularly inadequate in detecting rare or atypical pathogens.

Failure to promptly and accurately identify the causative organisms increases the risk of worsening infection and incomplete treatment. As a high-throughput, sensitive diagnostic technique, next-generation sequencing (NGS) has been widely applied in the diagnosis of pulmonary infections. Several studies have demonstrated that NGS offers superior sensitivity and detection rates compared to traditional methods[3, 4], and early pathogen-targeted therapy has been shown to significantly reduce mortality in patients with severe pneumonia[5]. Diao et al. even suggested that metagenomic next-generation sequencing (mNGS) may become a key diagnostic tool for infectious diseases in the coming decade[6]. Given the clinical complexity and diagnostic challenges in cases with disease progression or rare pathogen infections, repeat BALF NGS testing may be essential. This study retrospectively analyzes the pathogen distribution and sequence count changes in patients with severe pneumonia or complicated pulmonary infections who underwent two BALF NGS tests, aiming to provide evidence for precise antimicrobial therapy and therapeutic efficacy assessment.

Materials and methods

General information

This retrospective study included patients with severe pneumonia or complicated pulmonary infections who underwent two BALF NGS tests at the 924th Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army between January 2023 and September 2025.

Inclusion criteria were as follows: (1) They underwent BALF NGS testing based on clinical indications for complicated pulmonary infections, including: patients with severe pneumonia; patients presenting with prominent symptoms such as cough, expectoration, fever, or significant systemic toxic manifestations (e.g., fatigue, poor appetite, confusion, multiple organ dysfunction) who showed no response to 3–5 days of conventional anti-infective therapy and exhibited rapid disease progression; and patients with unexplained pulmonary opacities suspected of being infectious in etiology. All included patients were required to have relatively complete clinical data. (2) They provided consent for bronchoscopy and BALF NGS testing. (3) They underwent two consecutive BALF NGS tests during the course of treatment due to clinical deterioration or suspected infection with uncommon pathogens requiring efficacy evaluation, where conventional diagnostic methods failed to detect the causative microorganisms.

Exclusion criteria included: (1) intolerance to bronchoscopy; (2) missing key clinical data or parameters that precluded statistical analysis; (3) failure to undergo concurrent NGS and conventional etiological testing.

This study was approved by the Ethics Committee of the 924th Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army.

Data collection

General clinical data of patients were collected from the hospital's case management system, including sex, age, main symptoms, diagnosis, underlying diseases, reasons for undergoing two BALF NGS tests, disease severity classification, results of the first and second sputum examinations, bronchoalveolar lavage fluid (BALF) analysis, BALF NGS findings and pathogenic sequence counts, testing methods, and chest CT findings before and after treatment. Based on the ATS/IDSA criteria for severe pneumonia, patients were classified into severe and non-severe groups. The severe group was defined as patients meeting at least one major criterion or three or more minor criteria. Major criteria included the need for invasive mechanical ventilation or septic shock requiring vasopressors. Minor criteria comprised respiratory rate ≥30 breaths/min, PaO_2/FiO_2 ≤250, multilobar infiltrates, confusion/disorientation, azotemia, leukopenia, thrombocytopenia, and hypothermia. Patients who did not fulfill these criteria were assigned to the non-severe group. Additionally, based on the reason for undergoing a second BALF NGS test, patients were categorized into the clinical deterioration group and the routine follow-up group. Clinical deterioration was defined as worsening of symptoms, emergence of new symptoms, or CT findings indicating disease progression or lack of absorption.

BALF collection and NGS testing procedures

On days 1–2 following admission and completion of chest CT, selective fiberoptic bronchoalveolar lavage was performed in affected lobes or segments identified as newly involved on imaging. A total of 80–100 mL of sterile normal saline was instilled and aspirated to collect BALF samples using a suction

device. The BALF specimens were immediately placed in sterile tubes and sent for BALF mNGS or tNGS testing. A second BALF NGS test was performed in patients whose clinical condition worsened or in whom Uncommon Pathogens required follow-up to evaluate therapeutic efficacy; the sampling procedure was identical to the first.

Targeted NGS (tNGS)

Total nucleic acids were extracted from each specimen, and multiplex PCR primers were used to amplify target pathogen gene sequences. Library construction was carried out using a 300+ Pathogen Targeted Detection Kit (probe-anchored polymerization sequencing method, Digen Bio-technology Co., Ltd., Guangzhou, China), followed by sequencing on the MGISEQ-200 platform (MGI Tech Co., Ltd., China). Bioinformatic analysis focused exclusively on the targeted regions; pathogens were identified and their sequence reads quantified by alignment with a reference database. Relative quantification of pathogen concentration was achieved by comparing detected read counts against an internal standard of known concentration included in the library preparation reagents, with concentrations exceeding 100 copies/mL considered positive[7]. Final results were interpreted in conjunction with clinical manifestations, imaging findings, and conventional etiological tests to exclude potential contaminants or background flora.

Metagenomic NGS (mNGS)

Bronchoalveolar lavage fluid specimens were first processed to remove host cell debris, after which total nucleic acids were extracted. The extracted nucleic acids were randomly fragmented, adapter sequences were ligated, and following purification, a sequencing library was constructed. Shotgun sequencing was conducted on the MGISEQ-200 platform (MGI Tech Co., Ltd., China). After filtering the sequencing data, microbial species were identified, and their read counts and relative abundances were determined by alignment against a known microbial reference database [8]. Given the inherent limitations of NGS in definitively distinguishing pathogenic infection from colonization or contamination [9,10], result interpretation was guided by established expert consensus[11]. According to this consensus, a positive mNGS result was defined as follows: for bacteria or viruses, a microbial coverage rate at least 10-fold higher than that of other background microorganisms; for fungi, a coverage rate at least 5-fold higher than that of other background fungi, with Aspergillus species specifically requiring a strict threshold of ≥2 reads; for Mycobacterium tuberculosis, a threshold of ≥1 read was considered positive. The final determination of a causative pathogen required comprehensive integration of clinical findings, including sputum smear and culture results, chest CT findings, and medication history, in conjunction with the mNGS data.

Statistical analysis

To ensure comparability of pathogenic microorganisms across different samples, the sequence counts of pathogens detected by BALF NGS were normalized prior to analysis. The normalized sequence count was calculated as: (number of reads for a given species / clean reads) × 1M, where clean reads refer to

the effective sequencing data remaining after quality control filtering, and 1M represents 1,000,000 reads. Statistical analysis was performed using SPSS software (version 29.0). Continuous variables were expressed as mean ± standard deviation ($\bar{x} \pm s$) and compared using the t-test. Categorical variables were presented as counts and percentages [n (%)] and analyzed using the chi-square test. When the expected frequency was less than 5 in more than 20% of the cells, Fisher's exact test was applied. A significance level of $\alpha = 0.05$ was used for all statistical tests.

Results

General clinical characteristics of patients undergoing two BALF NGS tests

A total of 34 patients with severe pneumonia or complicated pulmonary infections who underwent two BALF NGS tests were included in this study. Among them, 26 (76.47%) were male and 8 (23.53%) were female. Twenty-one patients (61.76%) were aged over 60 years, while 13 (38.24%) were under 60 years of age. The most common clinical symptom was cough, reported in 28 patients (82.35%), followed by sputum production (58.82%), fever (35.29%), and shortness of breath or wheezing (41.18%). Less frequent symptoms included hemoptysis (5.88%) and chest pain (5.88%). Twenty-seven patients (79.41%) had at least one underlying comorbidity. The five most common comorbid conditions were respiratory failure (47.06%), chronic obstructive pulmonary disease (26.47%), bronchiectasis (23.53%), malignancy (20.59%), and chronic cardiac insufficiency (14.71%) (**Table 1)**.

Dynamic changes in pathogens detected by sequential BALF NGS testing

A total of 25 pathogen types were identified across the two BALF NGS tests. In the first test, pathogenic microorganisms were detected in 63 (188.24%) of the 34 patients by BALF NGS, compared with 9 (26.47%) by conventional sputum testing and 10 (29.41%) by conventional BALF testing. The detection rate of the first BALF NGS was significantly higher than that of conventional etiological methods($P < 0.05$). Among the detected pathogens, the predominant Gram-negative bacteria were *Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Haemophilus parainfluenzae*; the predominant Gram-positive bacteria were non-tuberculous mycobacteria (NTM), *Enterococcus faecium*, and *Staphylococcus aureus*; and the predominant fungi were Aspergillus spp., *Pneumocystis jirovecii*, and Candida spp. In the second test, pathogenic microorganisms were detected in 51 (150.0%) of the 34 patients by BALF NGS, compared with 8 (23.52%) by conventional sputum testing and 6 (17.64%) by conventional BALF testing. The detection rate of the second BALF NGS was also significantly higher than that of conventional etiological methods ($P < 0.05$). The predominant Gram-negative bacteria in the second test were *Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Acinetobacter baumannii*; the predominant Gram-positive bacteria were *Enterococcus faecium, Staphylococcus haemolyticus*, and Staphylococcus epidermidis; and the predominant fungi remained Aspergillus spp., *Pneumocystis jirovecii*, and Candida spp. From the first to the second round of testing, the total number of pathogens detected decreased by 12 (−19.0%). Ten pathogens showed increased detection frequency, fourteen decreased, and one remained unchanged. Specifically, *Klebsiella pneumoniae* increased in two cases

(33.33%), *Pseudomonas aeruginosa* in one case (20%), *Acinetobacter baumannii* in two cases (66.70%), *Haemophilus influenzae* in one case (33.33%), and *Enterococcus faecium* in one case (33.30%). In contrast, the number of cases with *Pneumocystis jirovecii* decreased by three (75%), decreased by four (66.70%), and *Aspergillus spp.* decreased by three (33.33%) (**Figure 1**).

Dynamic distribution of major pathogens in sankey diagram

As shown in the **Figure 2**, there was a moderate to significant increase in the number of cases with *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii, and Staphylococcus haemolyticus. In contrast,* Pneumocystis jirovecii*, NTM, and Aspergillus spp*. showed a marked decrease. Five newly identified pathogens were detected in the second test that were absent in the first test, including *Staphylococcus haemolyticus, Stenotrophomonas maltophilia, Ascosphaera assamensis, Mycobacterium tuberculosis, and Cryptococcus spp.*

Comparison between severe and non-severe patient groups

Patients were stratified into severe and non-severe groups for statistical analysis. Comparison of baseline characteristics between the two groups revealed statistically significant differences in underlying comorbidities (P = 0.010, Fisher's exact test) and types of pathogens detected (P = 0.019, Fisher's exact test). The severe group had a higher prevalence of comorbid conditions and was more frequently infected with fungi and rare pathogens, whereas NTM were more commonly detected in the non-severe group. There were no significant differences between the groups in terms of sex, age, or mixed infection status. Mixed infection was defined as infection involving two or more types of pathogens. Details are provided in **Table 2.**

Distribution of pathogens between severe and non-severe groups

Overall, patients in the severe group had a greater number of detected pathogens compared to the non-severe group. In particular, *Aspergillus spp.* was detected in 9 cases (20%), followed by Uncommon Pathogens in 5 cases (10%) and *Klebsiella pneumoniae* in 5 cases (10%), forming the main pathogen profile of the severe group. In contrast, pathogens in the non-severe group were more evenly distributed, with NTM detected in 5 cases (26.32%) and *Staphylococcus epidermidis* in 3 cases (15.83%) being the most common. The top five pathogens in the severe group were *Aspergillus spp*., Uncommon Pathogens, *Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. In the non-severe group, the most frequently detected pathogens were NTM, *Staphylococcus epidermidis, Haemophilus parainfluenzae, Talaromyces marneffei*, and *Pneumocystis jirovecii*. (**Figure 3**)

Analysis of indications for the second BALF NGS test

Among the 34 patients who underwent a second BALF NGS test, 18 cases (53%) were due to clinical deterioration or suspected disease progression, 11 cases (32%) were routine follow-up tests, 3 (9%) underwent repeat BALF NGS testing due to suspected tuberculosis or cross-infection, and 2 (6%) due to suspected drug resistance. (**Figure 4**)

Pathogen detection in the disease progression group vs. the re-examination group

Among the 34 patients who underwent a second BALF NGS test, the two most common indications were clinical deterioration and routine re-examination. A total of 18 patients (53%) were classified into the disease progression group. In this group, 23 newly detected pathogens were identified in the second test, with bacteria accounting for 60.86% (14/23). The bacterial spectrum was diverse, including Gram-negative bacteria such as *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, as well as Gram-positive bacteria such as *Staphylococcus spp*., *Streptococcus spp.*, and *Enterococcus spp*. Hospital-acquired pathogens constituted the core infectious agents. The most frequently observed findings were as follows: no new pathogen detected in 4 cases (22%), among which 3 patients were later diagnosed with organizing pneumonia; *Acinetobacter baumannii* in 4 cases (17%); *Klebsiella pneumoniae* in 2 cases (8.69%); *Pseudomonas aeruginosa* in 2 cases (8.69%); and human cytomegalovirus (HCMV) in 2 cases (8.69%) (**Figure 5**).

Pathogen profile in the re-examination group

A total of 11 patients underwent a second BALF NGS test as part of routine re-examination. Among these, 15 pathogens were identified based on their initial NGS results. Fungal infections—primarily from the *Aspergillus genus* and *Talaromyces marneffei*—accounted for 53.31%, while NTM accounted for 26.61%, making them the dominant pathogens in this group. Specifically, *Aspergillus fumigatus* and *Aspergillus flavus* were each detected in 3 cases, *Aspergillus terreus* in 1 case, and *Talaromyces marneffei* in 1 case. Among NTM, *Mycobacterium intracellulare–avium* complex was found in 2 cases and *Mycobacterium chelonae* in 1 case. Additionally, *Chlamydia psittaci* was detected in 2 cases, and *Pneumocystis jirovecii* in 1 case (**Figure 6**).

Changes in normalized sequence counts in the re-examination group

Among the 11 patients in the re-examination group, all 15 pathogens identified in the first BALF NGS test showed a notable decrease in normalized sequence counts in the second test. Specifically, 10 pathogens (66%) were no longer detected, while 5 pathogens (33%) showed a significant reduction in normalized sequence counts (**Table 3**).

Discussion

With the ongoing global trend of population aging, the incidence of pulmonary infections—particularly severe pneumonia—in elderly patients is rising. These individuals often present with multiple comorbidities and polypharmacy, including chronic respiratory conditions such as chronic obstructive pulmonary disease (COPD) and bronchiectasis[12, 13]. When clinical deterioration occurs, the inability to accurately identify the responsible pathogen becomes a major challenge. Therefore, accurately identifying potential pathogens and evaluating the therapeutic efficacy against Uncommon Pathogens is an urgent clinical issue that needs to be addressed. Repeat BALF NGS testing can largely resolve these clinical problems. This study analyzed the dynamic changes in respiratory pathogens in patients with pulmonary infections who underwent two BALF NGS tests, which holds significant clinical value for diagnosis, treatment, and therapeutic evaluation in such patients.

This study found that the pathogens detected in the first BALF NGS test were mainly *Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Haemophilus parainfluenzae* among Gram-negative bacteria; *Mycobacterium spp*. (NTM), *Enterococcus faecium*, and *Staphylococcus aureus* among Gram-positive bacteria; and *Aspergillus spp.*, P*neumocystis jirovecii*, and *Candida spp*. among fungi. These findings differ from the distribution characteristics of lower respiratory tract pathogens reported by Lingyu Jiang and Rujun Hong et al.[4, 14]. Possible reasons are as follows: 1) the patients included in this study had more underlying diseases and more severe conditions; 2) the pathogens identified in this study were determined based on a combination of clinical symptoms, laboratory findings, diagnoses, and medication history, whereas in the above studies, pathogens were determined based solely on NGS detection results. However, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Staphylococcus haemolyticus* showed a marked increase in the second test, indicating a higher risk of nosocomial infection. This suggests the need to strengthen hospital infection control measures to reduce the incidence of hospital-acquired infections, and also reminds clinicians to consider the possibility of nosocomial infections when a patient's condition changes during hospitalization.

In addition, we found that the severe group was predominantly infected with Gram-negative bacteria, fungi, and Uncommon Pathogens. This differs from the findings of Girish B. Nair et al., who reported that structural lung diseases were mainly associated with Gram-negative bacterial infections, while Streptococcus pneumoniae remained the leading cause of severe community-acquired pneumonia, followed by viruses and Uncommon Pathogens[15]. This discrepancy may be due to the fact that only patients who underwent two rounds of NGS testing were included in our study. Among the fungal infections in the severe group, *Aspergillus spp*. was the most frequently detected. During the prolonged course of antifungal treatment, some patients may develop secondary infections. According to Zhao Zhan et al., co-infection with fungi and bacteria is the most commonly observed mixed infection pattern. Additionally, there was a notable increase in the number of Uncommon Pathogens in the severe group, such as Chlamydia psittaci[16]. Jiamei Chen et al. reported that among 50 patients with psittacosis, early diagnosis was often challenging, leading to delays in treatment and poor infection control[17]. However, with the application of BALF NGS testing and targeted therapy, patients gradually improved. These findings support the notion that severe patients are at higher risk of rare infections.

Moreover, we found that in the disease progression group, the main pathogens identified in the second BALF NGS test were *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. However, in four patients, no pathogens were detected in the second test, and among them, three were later diagnosed with organizing pneumonia. These three patients had a history of malignancy, chemotherapy, or immunotherapy. Related studies have reported that secondary organizing pneumonia may occur after cancer, chemotherapy, or immunotherapy, and it is often misdiagnosed as pulmonary infection in clinical practice[18]. Therefore, when patients show clinical deterioration after precise antimicrobial treatment, clinicians should consider the possibility of organizing pneumonia, especially in those with a history of malignancy, to avoid unnecessary repeat BALF NGS testing and reduce the medical burden on patients.

It is noteworthy that in the disease progression group, certain pathogens with cell walls—such as *Ascosphaera assamensis, Mycobacterium tuberculosis*, and *Cryptococcus spp.*—were not detected in the first NGS test but were identified in the second. Related studies have shown that these pathogens possess thick cell walls, which make cell lysis difficult and reduce DNA extraction efficiency, potentially resulting in false-negative results[19]. It is often challenging to distinguish whether these are newly acquired infections or previously existing infections that were undetected in the initial NGS test. Multiple studies have reported that the sensitivity of mNGS is comparable to that of cryptococcal antigen (CrAg) testing, and combined testing can further improve the diagnostic sensitivity for pulmonary cryptococcosis[20, 21]. Other studies have indicated that the positive rate of NGS for detecting *Cryptococcus* is lower than that of serum cryptococcal capsular polysaccharide antigen[22]. Therefore, in this study, the detection of *Cryptococcus spp*. and *Mycobacterium tuberculosis* in the second NGS test may be attributed to technical limitations in cell wall disruption during the first test. Our findings also indicate that infections involving NTM, fungi, and Uncommon Pathogens were the main reasons for re-examination with a second BALF NGS test in the re-examination group. This is consistent with the findings of Rong Wang et al., who highlighted the advantage of NGS in detecting Uncommon Pathogens and mixed infections[23]. Additionally, a previous study reported a case in which pulmonary paragonimiasis was definitively diagnosed only after a third BALF NGS test, further illustrating the difficulty of detecting such pathogens using traditional methods and the necessity of repeat NGS testing[24]. When pathogen identification is initially achieved by NGS and targeted therapy is administered, it becomes even more difficult to determine whether subsequent clinical deterioration is due to drug-resistant strains or newly acquired infections.

Routine re-examinations are primarily used for regular monitoring of chronic infections or rare pathogen infections and serve to guide therapeutic efficacy evaluation. Over 85% of the re-examinations in this study were directly related to clinical status, highlighting the critical role of precision diagnostics in clinical decision-making. In the re-examination group, normalized sequence counts of pathogens in the second NGS test showed a marked decline compared to the first test, with some pathogens becoming undetectable. This may indicate clinical improvement or complete pathogen clearance. Xueling Fang reported a successful case in which changes in *adenovirus* sequence counts from cerebrospinal fluid NGS were used to monitor treatment response, suggesting that pathogen sequence levels may reflect

disease severity and treatment efficacy[25]. Moreover, the magnitude of the sequence count may help determine whether colonizing organisms or opportunistic pathogens are truly pathogenic. In other words, after anti-infective treatment, if the sequence count of a detected pathogen falls below a certain threshold, it may suggest that the organism is no longer clinically significant or pathogenic, and even support discontinuation of treatment. However, this hypothesis requires further validation through large-scale studies.

This study has several limitations. First, regarding study design, this was a retrospective analysis. Due to the relatively high cost of NGS and its clinical application threshold, the number of patients undergoing two BALF NGS tests was limited, resulting in a relatively small sample size. Furthermore, there was considerable individual variation in the timing of the second NGS test, with varying intervals between the two tests, which may have introduced bias into the dynamic observation of pathogen profiles and affected the stability and generalizability of the study findings. Second, concerning consistency of testing methods, different technical platforms were used for the two tests in some included cases. Specifically, some patients underwent mNGS for the first test and tNGS for the second test due to financial considerations or clinical necessity. Given the differences between tNGS and mNGS in terms of detection principles, coverage, sensitivity, and specificity, this inconsistency in testing methods may have introduced systematic errors in pathogen detection, thereby affecting the comparability of results between the two tests and the accuracy of treatment efficacy evaluation. Third, regarding data analysis and clinical assessment, the pathogenic microorganisms included in this study were diverse, encompassing bacteria, fungi, viruses, and other pathogen types. Due to significant differences in the biological characteristics, pathogenic mechanisms, and treatment responses among different pathogens, stratified or subgroup statistical analyses based on pathogen type were not feasible given the limited sample size, which to some extent constrained the depth of our findings. Additionally, when using the two NGS results for efficacy evaluation, there was a lack of unified objective criteria, and some assessments may have been influenced by the subjective experience of clinicians, introducing a degree of subjectivity. In view of these limitations, future research should focus on specific pathogen types (e.g., Aspergillus spp.) through large-sample, prospective cohort studies, with unified testing methods and standardized timing of tests incorporated into the study design. Such efforts would provide more reliable evidence-based medical evidence for treatment efficacy evaluation in patients with pulmonary infections.

Conclusion

This study performed a dynamic analysis of the pathogenic spectrum in 34 patients with severe pneumonia or complicated pulmonary infections who underwent two consecutive BALF NGS tests. The results showed that in the second test, the detection counts of *Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Staphylococcus haemolyticus* increased significantly, suggesting that these pathogens may be closely associated with disease progression or secondary infection. Further analysis revealed that patients in the severe group had a greater burden of underlying diseases compared with those in the non-severe group, and their pathogenic spectrum was

predominantly composed of fungi and atypical or opportunistic pathogens, whereas non-tuberculous mycobacteria (NTM) were the main pathogen type in the non-severe group. Additionally, in the clinical deterioration group, the primary causative pathogens of new infections were *Acinetobacter baumannii, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. In contrast, infections with NTM, fungi, and atypical or opportunistic pathogens served as the main indications for undergoing a second BALF NGS test in the routine follow-up group. These findings provide important reference for identifying potential pathogens and guiding adjustments to anti-infective therapy when clinical deterioration occurs in patients with severe pneumonia or complicated pulmonary infections in clinical practice, thereby avoiding unnecessary repeat BALF NGS testing and reducing the financial burden on patients. More critically, in the routine follow-up group, the normalized sequence counts of certain pathogens decreased significantly or even became undetectable compared with the first test. This trend may serve as an objective molecular biomarker for evaluating treatment efficacy against specific pathogens (particularly difficult-to-treat or atypical pathogens such as fungi and NTM), suggesting potential clinical application value.

Abbreviations

NGS, Next-generation sequencing;

BALF, Bronchoalveolar lavage fluid;

NTM, Non-tuberculous mycobacteria;

BALF NGS, Bronchoalveolar lavage fluid metagenomic next-generation sequencing;

Declarations

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki. The study protocol was reviewed and approved by the Ethics Committee of the No. 924 Hospital of the Joint Logistics Support Force of the Chinese People's Liberation Army (approval number: [2025]159). Written informed consent was obtained from all individual participants included in the study.

Consent to Publication

Not applicable

Clinical trial number

not applicable

Data availability statement

The datasets generated during and/or analyzed during the current study are not publicly available due to ethical and privacy restrictions, but may be available from the corresponding author upon reasonable request and with approval from the institutional ethics committee.

Competing interests

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.

Funding

Not applicable.

Author contributions

Junzhang Qin: Writing – original draft and Data curation. **Xiandong Yin**: Writing – review & editing and Conceptualization. **Zhen Xiong**: Project administration and Investigation. All authors have read and approved the final version of the manuscript.

Acknowledgments

We sincerely th8ank all participants for their cooperation and contribution to this clinical study.

References

1. Aston S J, Wootton D G. Community-acquired pneumonia due to drug-resistant enterobacteriaceae: a global perspective[J]. Respirology, 2020, 25(5): 468-469.
2. Metlay J P, Waterer G W, Long A C, et al. Diagnosis and treatment of adults with community-acquired pneumonia. An official clinical practice guideline of the american thoracic society and infectious diseases society of america[J]. American Journal of Respiratory and Critical Care Medicine, 2019, 200(7): e45-e67.
3. Jin X, Li J, Shao M, et al. Improving suspected pulmonary infection diagnosis by bronchoalveolar lavage fluid metagenomic next-generation sequencing: a multicenter retrospective study[J]. Microbiology Spectrum, 2022, 10(4): e02473-21.
4. Jiang L, Han L, Zhong Y, et al. High utility of bronchoalveolar lavage fluid metagenomic next-generation sequencing approach for etiological diagnosis of pneumonia[J]. BMC infectious diseases, 2024, 24(1): 1232.
5. Seymour C W, Gesten F, Prescott H C, et al. Time to treatment and mortality during mandated emergency care for sepsis[J]. New England Journal of Medicine, 2017, 376(23): 2235-2244.

6. Z D, D H, R Z, et al. Metagenomics next-generation sequencing tests take the stage in the diagnosis of lower respiratory tract infections[J]. Journal of Advanced Research, 2022,38(c):201-212.
7. Chao L, Li J, Zhang Y, et al. Application of next generation sequencing-based rapid detection platform for microbiological diagnosis and drug resistance prediction in acute lower respiratory infection[J]. Annals of Translational Medicine, 2020, 8(24): 1644.
8. Peng J M, Du B, Qin H Y, et al. Metagenomic next-generation sequencing for the diagnosis of suspected pneumonia in immunocompromised patients[J]. Journal of Infection, 2021, 82(4): 22-27.
9. Chen J, Qin Z, Jia Z. The application status of sequencing technology in global respiratory infectious disease diagnosis[J]. Infection, 2024, 52(6): 2169-2181.
10. Liu H, Zhang Y, Yang J, et al. Application of mNGS in the etiological analysis of lower respiratory tract infections and the prediction of drug resistance[J]. Microbiology Spectrum, 2022, 10(1): e0250221.
11. Chinese Thoracic Society, Chinese Association of Chest Physicians. [Expert consensus on the clinical interpretation pathway for metagenomic next-generation sequencing reports of lower respiratory tract infections]. Zhonghua Jie He He Hu Xi Za Zhi. 2023;46(4):322-335.
12. Govender K N, Street T L, Sanderson N D, et al. Metagenomic sequencing as a pathogen-agnostic clinical diagnostic tool for infectious diseases: a systematic review and meta-analysis of diagnostic test accuracy studies[J]. Journal of Clinical Microbiology, 2021, 59(9): e0291620.
13. Mancini N, Carletti S, Ghidoli N, et al. The era of molecular and other non-culture-based methods in diagnosis of sepsis[J]. Clinical microbiology reviews, 2010, 23(1): 235-251.
14. Hong R, Lin S, Zhang S, et al. Pathogen spectrum and microbiome in lower respiratory tract of patients with different pulmonary diseases based on metagenomic next-generation sequencing[J]. Frontiers in Cellular and Infection Microbiology, 2024 Oct 31:14:1320831.
15. Nair G B, Niederman M S. Updates on community acquired pneumonia management in the ICU[J]. Pharmacology & Therapeutics, 2021 Jan;217:107663.
16. Zhao Z, Song J, Yang C, et al. Prevalence of fungal and bacterial Co-infection in pulmonary fungal infections: a metagenomic next generation sequencing-based study[J]. Frontiers in Cellular and Infection Microbiology, 2021 Nov 1;11:749905.
17. Chen J, Wang J, Deng Z, et al. Clinical features of 50 cases of chlamydia psittaci pneumonia identified through metagenomic next-generation sequencing[J]. Infection and Drug Resistance, 2024 Dec 21;17:5775-5784.
18. Limkul L, Tovichien P. Secondary organizing pneumonia after infection[J]. World Journal of Clinical Cases, 2024, 12(36): 6877-6882.
19. Chiu C Y, Miller S A. Clinical metagenomics[J]. Nature Reviews Genetics, 2019, 20(6): 341-355.
20. Huang J, Weng H, Ye L, et al. Bronchoalveolar lavage fluid and lung biopsy tissue metagenomic next-generation sequencing in the diagnosis of pulmonary cryptococcosis[J]. Frontiers in Cellular and Infection Microbiology, 2024 Oct 29;14:1446814.

21. Yan Q F, Sun Z L, Gao Y, et al. [Diagnostic value of the combinations of bronchoalveolar lavage fluid pathogen detection and cryptococcal antigen test in pulmonary cryptococcosis][J]. Zhonghua Jie He He Hu Xi Za Zhi = Zhonghua Jiehe He Huxi Zazhi = Chinese Journal of Tuberculosis and Respiratory Diseases, 2021, 44(8): 711-716.
22. Miao Q, Ma Y, Wang Q, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice[J]. Clin Infect Dis. 2018 Nov 13;67(suppl_2): S231-S240.
23. Wang R, Wang H, Jiang J, et al. Diagnostic value of metagenomic next-generation sequencing in the etiological diagnosis of lower respiratory tract infection[J]. Scientific Reports, 2025, 15(1): 39987.
24. Qin J, Zhu W, Xiong Z. High-throughput sequencing of bronchoalveolar lavage fluid confirms pulmonary paragonimiasis: A case report[J]. Medicine, 2025, 104(43): e45261.
25. Xueling Fang, Xu M, Fang Q, et al. Real-time utilization of metagenomic sequencing in the diagnosis and treatment monitoring of an invasive adenovirus B55 infection and subsequent herpes simplex virus encephalitis in an immunocompetent young adult[J]. Open Forum Infectious Diseases, 2018, 5(6): ofy114.

Tables

Table 1 Demographic and clinical characteristics of patients underwent two BALF NGS tests

Characteristics	Number (n = 34)	Proportion (%)
Gender		
Male	26	76.47
Female	8	23.53
Age		
>60 years	21	61.76
≤60 years	13	38.24
Clinical Manifestations		
Cough	28	82.35
Sputum production	20	58.82
Fever	12	35.29
Shortness of breath / Wheezing	14	41.18
Chest pain	2	5.88
Hemoptysis	2	5.88
Underlying Comorbidities	27	79.41%
Respiratory failure	16	47.06
COPD	9	26.47
Bronchiectasis	8	23.53
Malignancy	7	20.59
Chronic cardiac insufficiency	5	14.71
Pulmonary fibrosis	1	2.94
Type 2 diabetes mellitus	2	5.88
Bronchial asthma	1	2.94
Hypertension	2	5.88

COPD, Chronic obstructive pulmonary disease.

Table 2 Comparison of baseline characteristics between severe and non-severe groups

Characteristic	Severe Group (n = 24)	Non-Svere Group (n = 10)	Statistic	P-value
Age	65.04 ± 15.50	55.60± 12.39	t = 1.75	0.09
Gender			Fisher	0.67
Male	19 (79.16)	7 (70)		
Female	5 (20.83)	3 (30)		
Comorbidities	22 (91.66)	5 (50)	Fisher	0.01
Co-infection	10 (41.60	3 (30	Fisher	0.70
Total Pathogens Detected	50	19	Fisher	0.02
Bacteria	25 50	10 52.63		
Fungi	16 32	4 21.05		
NTM	1 2	5 26.32		
Virus	3 6	0 0		
Uncommon Pathogens	5 10	0 0		

NTM, Non-tuberculous mycobacteria. Fisher's exact test was used because the expected frequency was <5 in >20% of the cells.

Table 3 Changes in normalized sequence counts of pathogens in the follow-up group

Number	Pathogen	Normalized Sequence Count (1st Test)	Normalized Sequence Count (2nd Test)
1	*Aspergillus fumigatus*	19840	0
	Aspergillus flavus	281	0
3	MAC	9	2
15	*Aspergillus fumigatus*	310	0
	Aspergillus flavus	1989	0
18	*Talaromyces marneffei*	1537.58	0
20	*Pneumocystis jirovecii*	414	0
21	*Mycobacterium chelonae*	130	0
24	*Mycobacterium avium*	25	0
27	*Aspergillus fumigatus*	6746	5566
	Aspergillus flavus	26465	21839
	Aspergillus terreus	474	0
30	*Mycobacterium abscessus*	3222	2492
31	*Chlamydia psittaci*	949794	12704
33	*Chlamydia psittaci*	717988	10003

MAC, Mycobacterium avium complex.

Figures

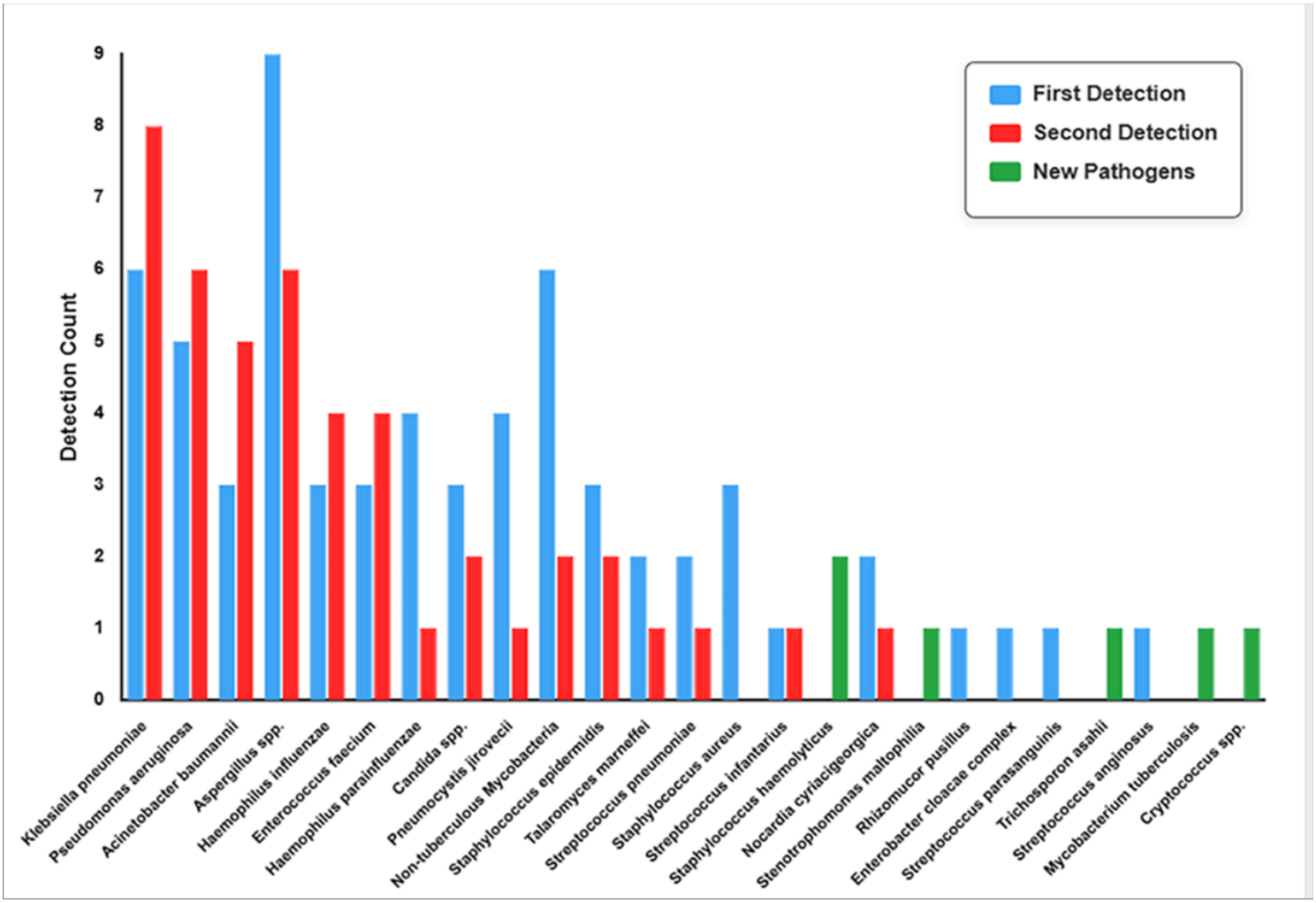


Figure 1

Legend not included with this version.

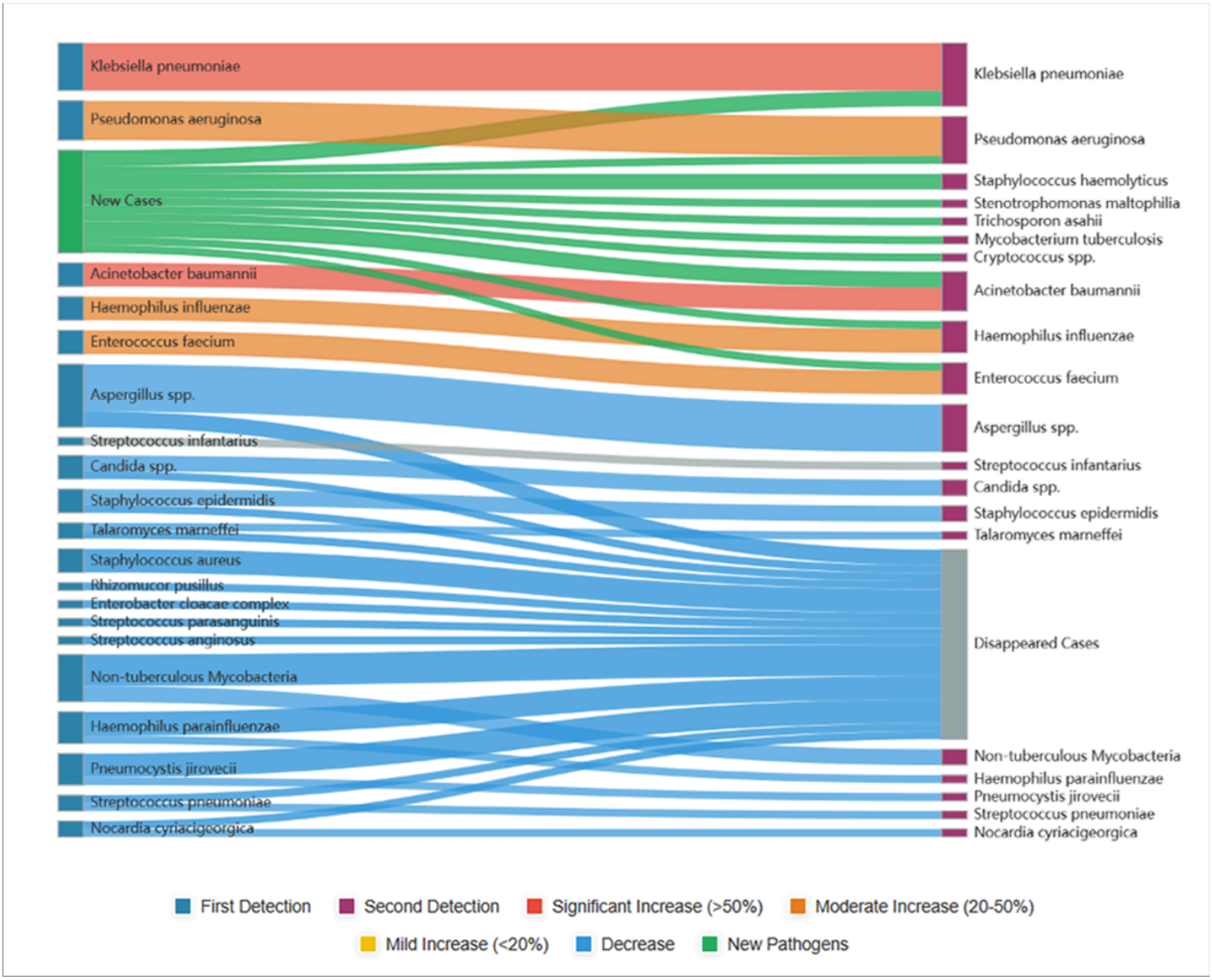


Figure 2

Legend not included with this version.

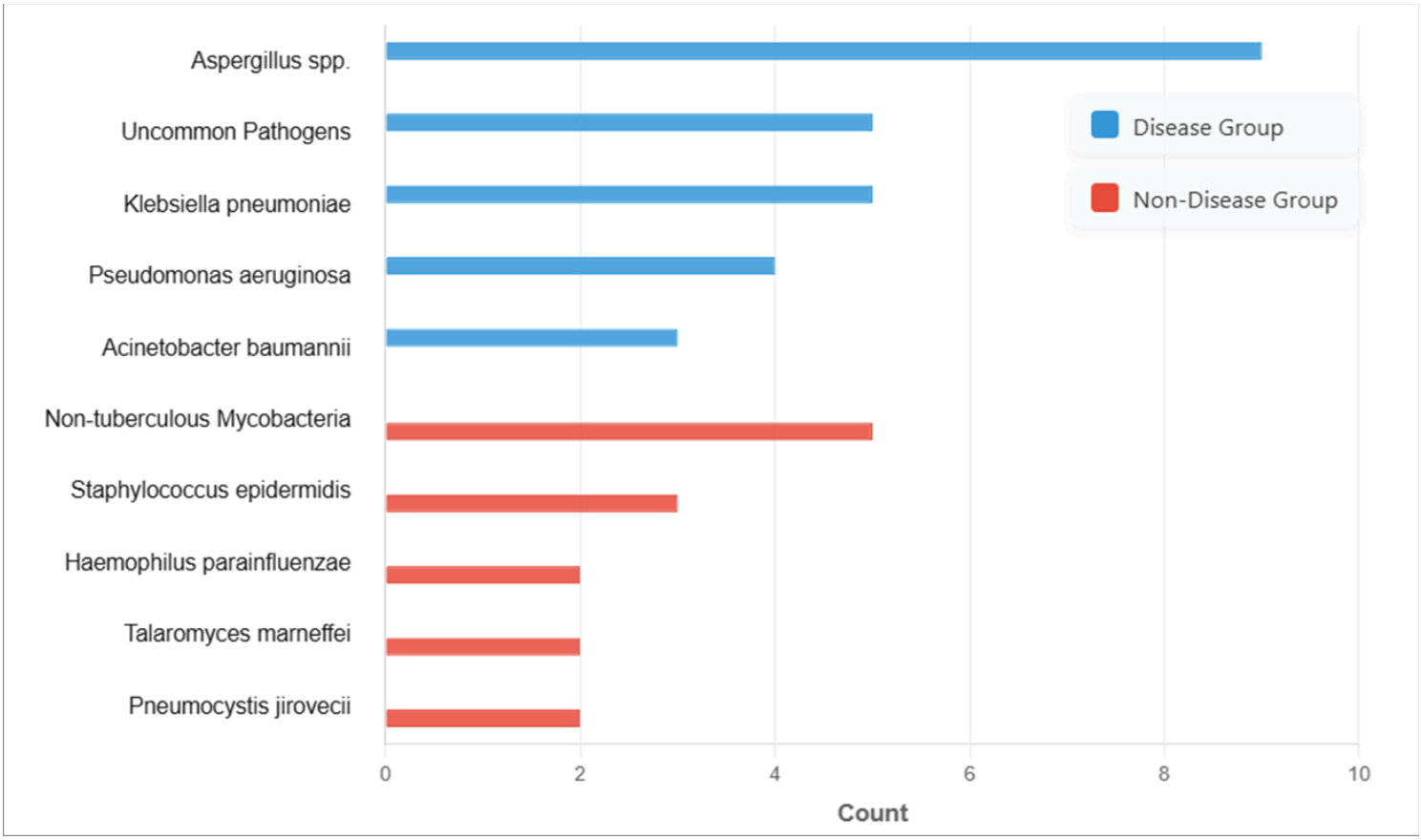


Figure 3

Legend not included with this version.

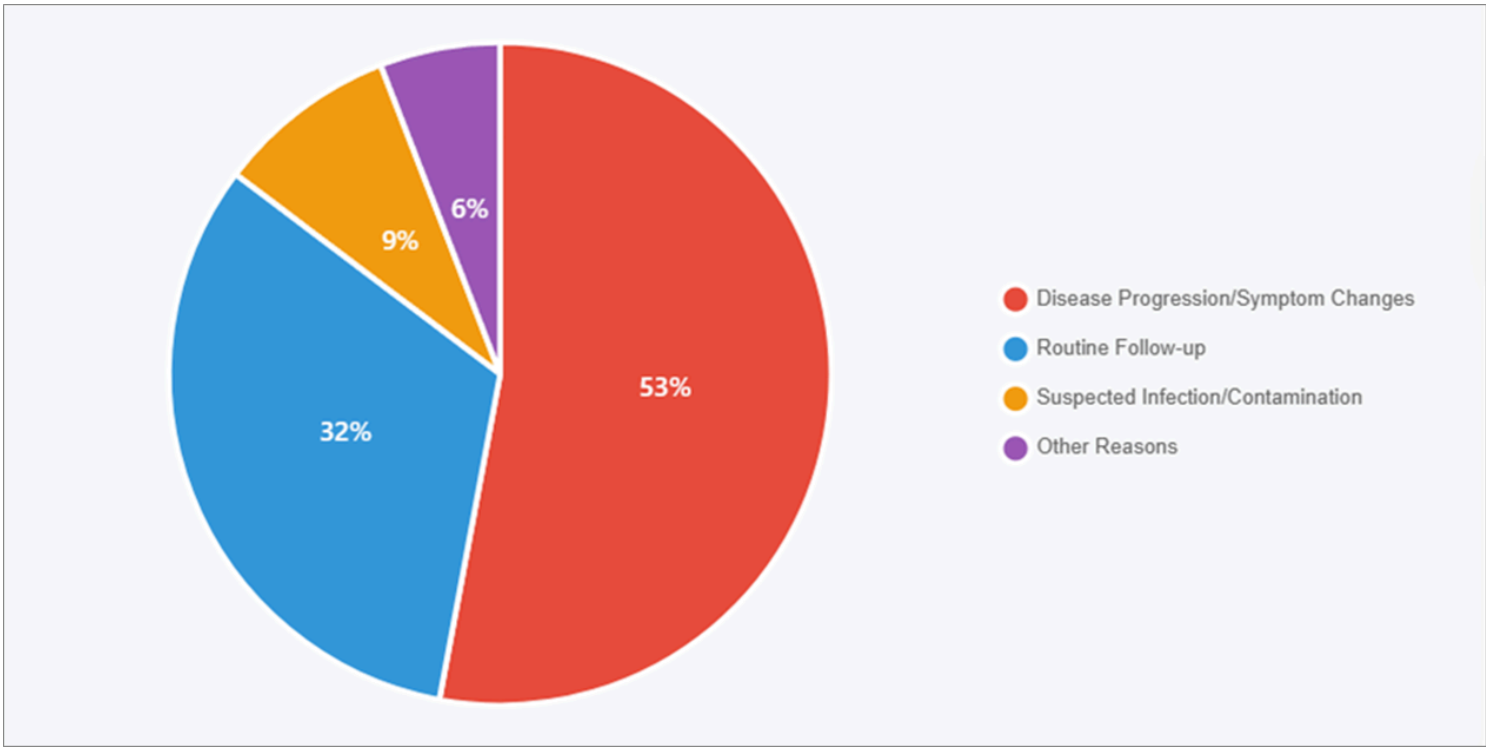


Figure 4

Legend not included with this version.

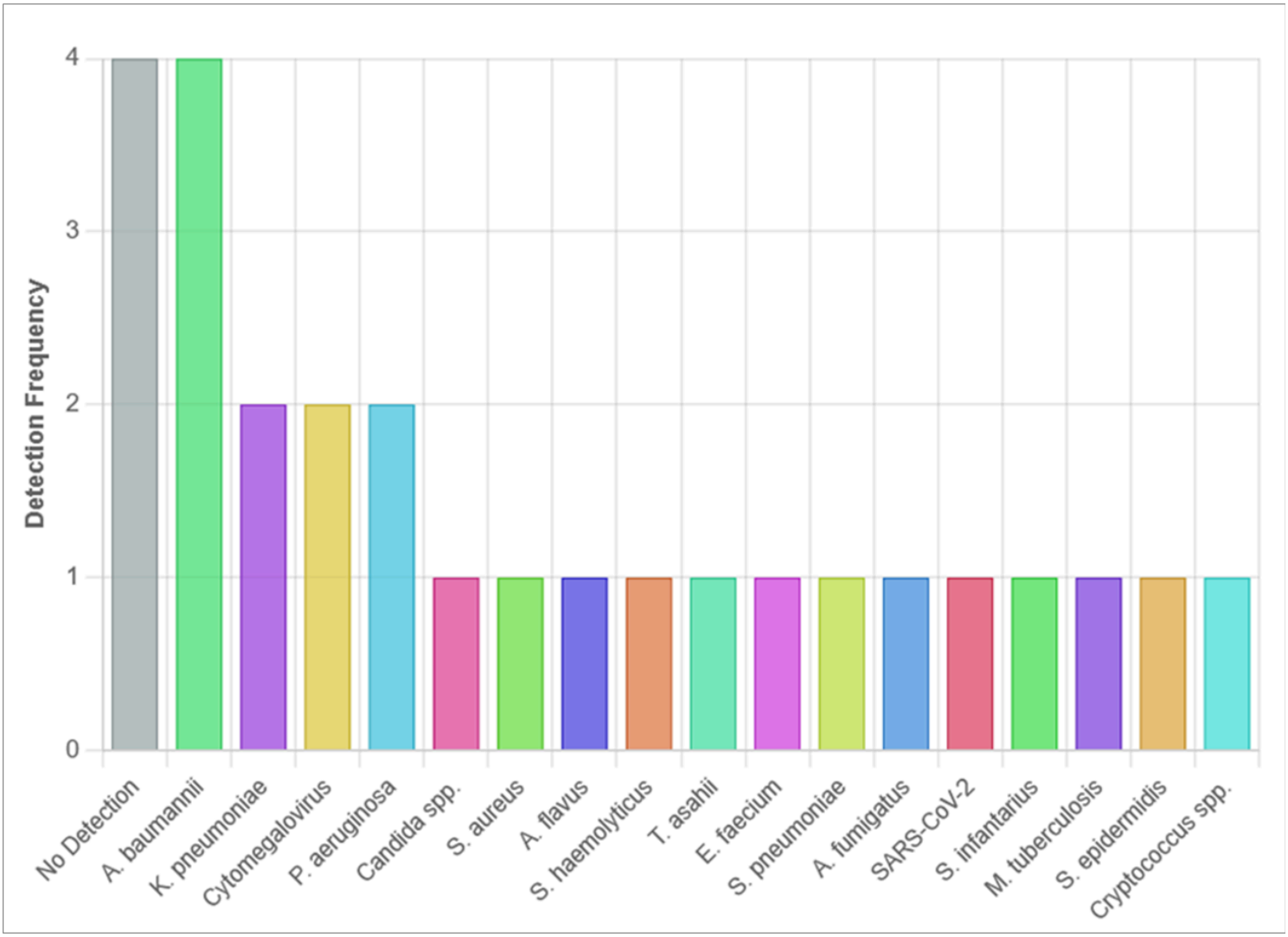


Figure 5

Legend not included with this version.

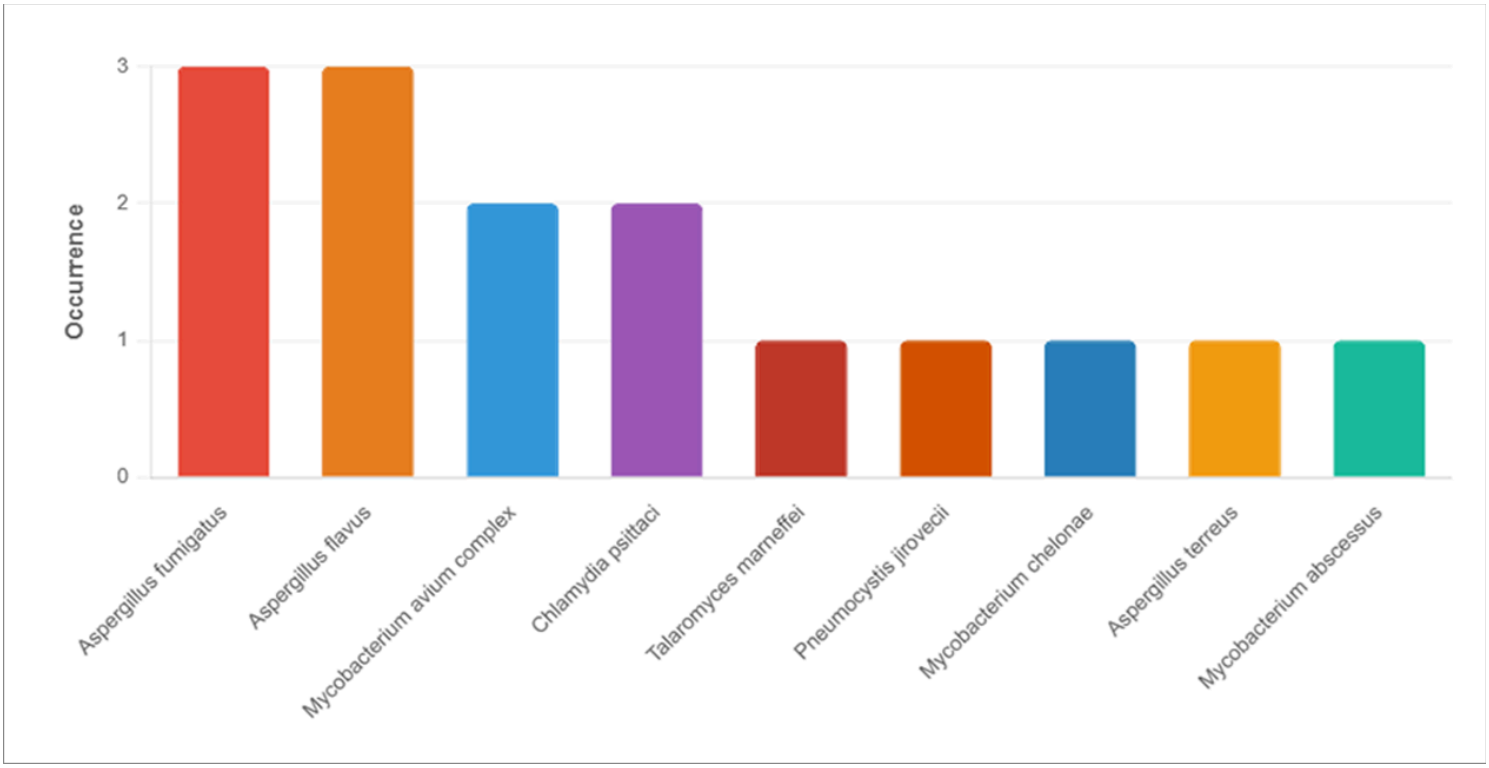


Figure 6

Legend not included with this version.