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Evaluation of Different Sampling Methods Combined with Metagenomic Next-Generation Sequencing of Respiratory Specimens in Etiological Diagnosis of Patients with Severe Pneumonia

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Objective: To evaluate the value of respiratory specimens collected via different sampling methods combined with metagenomic next-generation sequencing (mNGS) in the etiological diagnosis of severe pneumonia.

Methods: A total of 117 patients with severe pneumonia between 2019 and 2024 were included in this study, with 60 patients undergoing endotracheal aspiration (ETA) and 57 undergoing bronchoalveolar lavage (BAL), respectively. Patient records were retrospectively reviewed. Both ETA and BAL samples were tested using mNGS and conventional microbiological tests (CMT) to compare the detection rates, microbial profiles and their effects on clinical outcomes.

Results: The positive rates of mNGS for ETA and BAL samples were 96.7% and 80.7%, respectively, which were higher than CMT. A total of 39 pathogenic microorganisms were detected, of which *Klebsiella pneumoniae, Candida albicans* and *herpes simplex virus*-4 (HSV-4), and *cytomegalovirus* (CMV) were the most commonly detected as bacteria, fungi and viruses, respectively. The percentages of *Pseudomonas aeruginosa* (30.0% vs 12.3%, $p = 0.019$) and *Stenotrophomonas maltophilia* (25.0% vs 8.8%, $p = 0.020$) were significantly higher in the ETA group compared to the BALF group. The detection rate of three or more microorganisms was notably higher in the ETA group. No significant differences existed in antibiotics adjustment between the groups. The ETA group experienced a higher frequency of continuous renal replacement therapy (CRRT), mechanical ventilation and complications. There was no significant difference in the hospital length of stay, duration of mechanical ventilation and mortality between both groups.

Conclusion: Respiratory specimens collected by different sampling methods yield different microbial findings. ETA and BAL combined with mNGS play a role in guiding the pathogenetic diagnosis of patients with severe pneumonia. However, it is recommended that their sampling methods be determined by clinical symptoms and patient conditions.

Keywords: metagenomic next-generation sequencing, sampling method, severe pneumonia, etiological diagnosis

Introduction

Pneumonia is a common clinical respiratory infectious disease, especially severe pneumonia, which has been on the rise in recent years, with a high morbidity and mortality rate, contributing significantly to familial and societal burdens.[1,2] Pneumonia is an inflammatory response of the lung triggered by varied pathogens, necessitating swift identification of its etiological agents to initiate effective antibiotic treatment and decrease fatality rates.[3–5]

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In recent years, the maturity and popularization of mNGS technology provide a new powerful means for etiological diagnosis. It does not need to be cultured, and a small amount of DNA extracted from the sample can be detected, with high identification speed and high sensitivity of pathogen identification. This technique not only supplements conventional pathogen detection but also augments the clinical pathogen detection rate, offering distinct advantages in directing the diagnosis and anti-infective therapy of severe infection patients.[6,7] mNGS is a comprehensive tool to assist in the diagnosis of pathogens in lower respiratory tract infections.[8]

Common microbiology specimens for respiratory infections encompass sputum, lung tissue, BAL and ETA. Previous studies have shown that the distribution of pathogen components is different due to different sampling methods. BAL samples were more sensitive and specific to pathogen detection than sputum samples.[9] Obtaining BAL samples necessitates the expertise and experience of clinical physicians, making the process relatively time-consuming and intricate. Given that most patients with severe pneumonia undergo endotracheal intubation and mechanical ventilation, tracheal aspirates are frequently employed as a secondary choice for respiratory clinical specimens, owing to their ease of access. At present, there are no clear recommendations for microbiological diagnostic specimens of severe pneumonia. There are few reports on the application of ETA and BAL combined with mNGS for pathogen detection in pulmonary infection. In this study, we retrospectively analyzed ETA and BAL samples combined with mNGS from patients with severe pneumonia in our hospital, so as to investigate the impact of different sampling methods on the pathogenic diagnosis, treatment and prognosis of patients with severe pneumonia.

Materials and Methods

Study Population

This retrospective study was conducted at Taizhou Hospital of Zhejiang Province and recruited patients hospitalized for suspected pneumonia from January 2019 to February 2024. All patients met the American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA 2019) criteria for predicting severe pneumonia. Inclusion criteria included: (1) diagnosis of severe pneumonia, (2) respiratory specimens were examined with both mNGS and CMT[10]. Exclusion criteria included: (1) age <18 years, (2) other testing methods were used to identify patients with severe pneumonia infected with COVID-19, (3) transfer to another hospital or incomplete information. Conventional microbiological tests included microbial culture, nucleic acid detection by PCR and antigen detection, which were reported in previous study.[10] Written informed consent was obtained from all patients, the study protocol complied with the principles of the Helsinki Declaration and was approved by the Ethics Committee of Taizhou Hospital of Zhejiang Province.

Clinical Data Collection

The following clinical data of each patient were collected from the electronic medical record, including basic information of the patient, such as age, gender, underlying disease and outcome, clinical laboratory data at enrollment and microbiological information, medication information (mainly antimicrobial therapy), and information on the patient treatment, mainly referring to whether mechanical ventilation was performed.

mNGS Procedure for Respiratory Specimens

Sample processing: samples were collected in strict accordance with clinical practice. ETA specimens: non-invasive method was used to collect samples by endotracheal suction. BAL specimens: for diffuse lung lesions, the middle lobe of the right lung (B4 or B5) or the lingual segment of the left lung was selected. For restrictive lung lesions, the segment of the bronchus with severe lesions was selected. After the tip of the bronchoscope was embedded in the appropriate branch of the bronchial tree, 60–120 mL of saline was injected with the recovery rate of 40–60%, and the recovered lavage fluid was filtered into a sterilized container. Specimens were collected and immediately sent to the laboratory for microbiological analysis.

mNGS sequencing and analysis were performed by the methods reported in the previous literature.[10] (1) Nucleic acid extraction: using nucleic acid extraction and purification kit (RM0184, BGI, China) to extract DNA. Only when the DNA concentration >0.1ng/μL can the next step be carried out. (2) DNA library was constructed through DNA-fragmentation,

end-repair, adapter-ligation and PCR amplification. Agilent 2100 was used for quality control of the DNA libraries to make sure that the size of fragments reached up to 300bp. Quality qualified libraries were pooled, DNA Nanoball (DNB) was made and sequenced by MGISEQ-2000 platform. DNB concentration ≥8ng/μL was considered as passing quality control. For each batch of products, we use positive and negative quality control products to control and minimize pollution. Q30 of each lane or sample should be 85% or above. The sequencing reads of each sample should be 20 million or above, and sequencing reads of internal standard should ≥3. (3) Bioinformatics analysis: remove low-quality data after sequencing data were taken off to obtain high-quality data. The data of human reference genome sequence were removed by comparison. The remaining data are compared with the special microbial database and classified and arranged according to bacteria, fungi, viruses and parasites. Interpretation criteria referred to in previous research.[11]

Clinical Impact Evaluation of mNGS

Clinical impacts of mNGS were determined by the impact on clinical management through reviewing electronic medical record system. Clinical factors included clinical manifestations, laboratory test indicators, imaging examinations, and treatment response. Retrospective assessment of the adjustment of anti-infective drugs and the outcome of severe pneumonia by clinical physicians based on clinical data and mNGS results.

Statistical Analysis

Normally distributed continuous variables were described using mean ± SD, while non-normally distributed continuous variables were displayed using median and interquartile range (IQR). Categorical variables were expressed as numbers and percentages of subjects. When comparing continuous variables, *Student's t*-test or *Mann–Whitney U*-test was used, and categorical variables were assessed through *chi-square's* test or *Fisher's exact* test. All statistical tests used were two-sided, and significance was defined as *p*-values less than 0.05. All statistical analyses were conducted using SPSS 23.0 (SPSS Inc., Chicago, IL, USA), and figures were generated using GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

A total of 117 patients with severe pneumonia were enrolled, including 85 males and 32 females, with an average age of 67.1 ± 14.7. According to different sampling methods, the participants were divided into two groups: ETA group (n = 60) and BAL group (n = 57). The demographics data, characteristic baselines, and laboratory examination results before treatment were presented in Tables 1 and 2. According to the clinical characteristics, underlying diseases and laboratory results were basically matched between the two groups. There was no significant difference in age, gender, smoking, drinking, underlying diseases, APACHE II score before treatment between the two groups. Moreover, no significant difference was found in laboratory indexes related to inflammation among different groups on admission.

The species distribution of bacteria, fungi, viruses and atypical pathogens were detected by mNGS, see Figure 1. *K. pneumoniae, C. albicans*, HSV-4 and CMV were the most commonly detected bacteria, fungi and viruses, respectively. *K. pneumoniae, Acinetobacter baumannii, P. aeruginosa* and *S. maltophilia* were the most frequently detected bacteria. The most commonly detected fungi were *C. albicans, Pneumocystis jiroveci*, and *Candida parapsilosis*. HSV-4, CMV and *Torque teno Virus* were the top three of the most detected viruses as determined by mNGS. Specifically, *P. aeruginosa* (30.0% vs 12.3%, $p = 0.019$) and *S. maltophilia* (25.0% vs 8.8%, $p = 0.020$) were more frequently detected in the ETA group compared with that in the BALF group. However, no significant differences were observed in the detected rates of fungi, viruses and atypical pathogens.

In the ETA group, there were 58 positive cases and 2 negative cases, the positive rate of mNGS was 96.7%, which was higher than that of CMT (45.0%). Additionally, mNGS revealed that there were 46 positive and 11 negative cases in the BAL group, resulting in a positive rate of 80.7%. The positive rate of the ETA group was significantly higher than that of the BAL group ($p = 0.006$), as shown in Figure 2.

Table 1 Demographics, Clinical Characteristics of Patients with Severe Pneumonia

Characteristics	ETA group (n = 60)	BAL group (n = 57)	*p* value
Age (years)	66.0±16.5	68.1±12.5	0.440
Gender, n(%)			0.865
Male	44(73.3)	41(71.9)	
Female	16(26.7)	16(28.1)	
Smoking status, n(%)			0.195
Non-smoker	38(63.3)	28(49.1)	
Ex-smoker	15(25.0)	16(28.1)	
Current smoker	7(11.7)	13(22.8)	
Drinking, n(%)	12(20.0)	14(24.6)	0.553
Symptoms, n(%)			
Dyspnea	6(10.0)	4(7.0)	0.744
Frailty	5(8.3)	4(7.0)	1.000
Cough	31(51.7)	37(64.9)	0.147
Chest distress	29(48.3)	24(42.1)	0.499
Fever	31(51.7)	28(49.1)	0.783
Obnubilation	4(6.7)	4(7.0)	1.000
Underlying conditions, n(%)			
Hypertension	30(50.0)	24(42.1)	0.392
Diabetes mellitus	10(16.7)	11(19.3)	0.711
Cardiovascular diseases	28(46.7)	21(36.8)	0.282
Chronic respiratory diseases	14(23.3)	14(24.6)	0.876
Malignancy	9(15.0)	11(19.3)	0.537
Immunosuppression	24(40.0)	16(28.1)	0.174
APACHE II score, median (IQR)	17.0(13.0–20.0)	16.0(12.0–18.5)	0.267
Antibiotics used before specimen collection	26(43.3)	23(40.4)	0.744

Notes: Chronic respiratory diseases: Asthma, Cystic fibrosis, Obsolete Pulmonary Tuberculosis.
Abbreviations: COPD, Chronic Obstructive Pulmonary Disease.

Table 2 Laboratory Findings of Patients with Severe Pneumonia

Laboratory findings	ETA group (n = 60)	BAL group (n = 57)	*p* value
WBC (10^9/L)	10.4(6.8–15.4)	10.1(6.0–12.5)	0.468
NEU%	90.8(83.7–94.1)	90.4(85.1–94.2)	0.915
CRP (mg/L)	134.2(69.5–218.9)	133.1(51.5–238.4)	0.581
PCT (ng/mL)	2.0(0.4–7.8)	0.5(0.2–8.9)	0.535
LDH (U/L)	388.0(277.8–533.3)	396.0(261.5–534.5)	0.824
FIB (g/L)	4.8(3.6–7.1)	5.3(4.2–6.8)	0.758
PaO_2/FiO_2 (mmHg)	160.5(112.8–222.8)	170.0(132.5–232.5)	0.586
Lac (mmol/L)	1.9(1.3–2.8)	2.4(1.7–3.4)	0.602

Abbreviations: WBC, white blood cell count; NEU%, neutrophil percentage; CRP, C-reactive protein; PCT, procalcitonin; LDH, lactate dehydrogenase; FIB, Fibrinogen; Lac, Lactic acid.

Among the ETA positive cases, 22 cases were found to have a single pathogen, accounting for 36.7%. Sixteen cases of two pathogens were detected, accounting for 26.7%. Three or more pathogens were detected in 20 cases, accounting for 33.3%. One, two, three or more pathogens were detected in BAL positive cases, accounting for 29.8%, 36.8% and 14.0%, respectively. The detection rate of three or more pathogens in the ETA group was significantly higher than that in the BAL group, as shown in Figure 3.

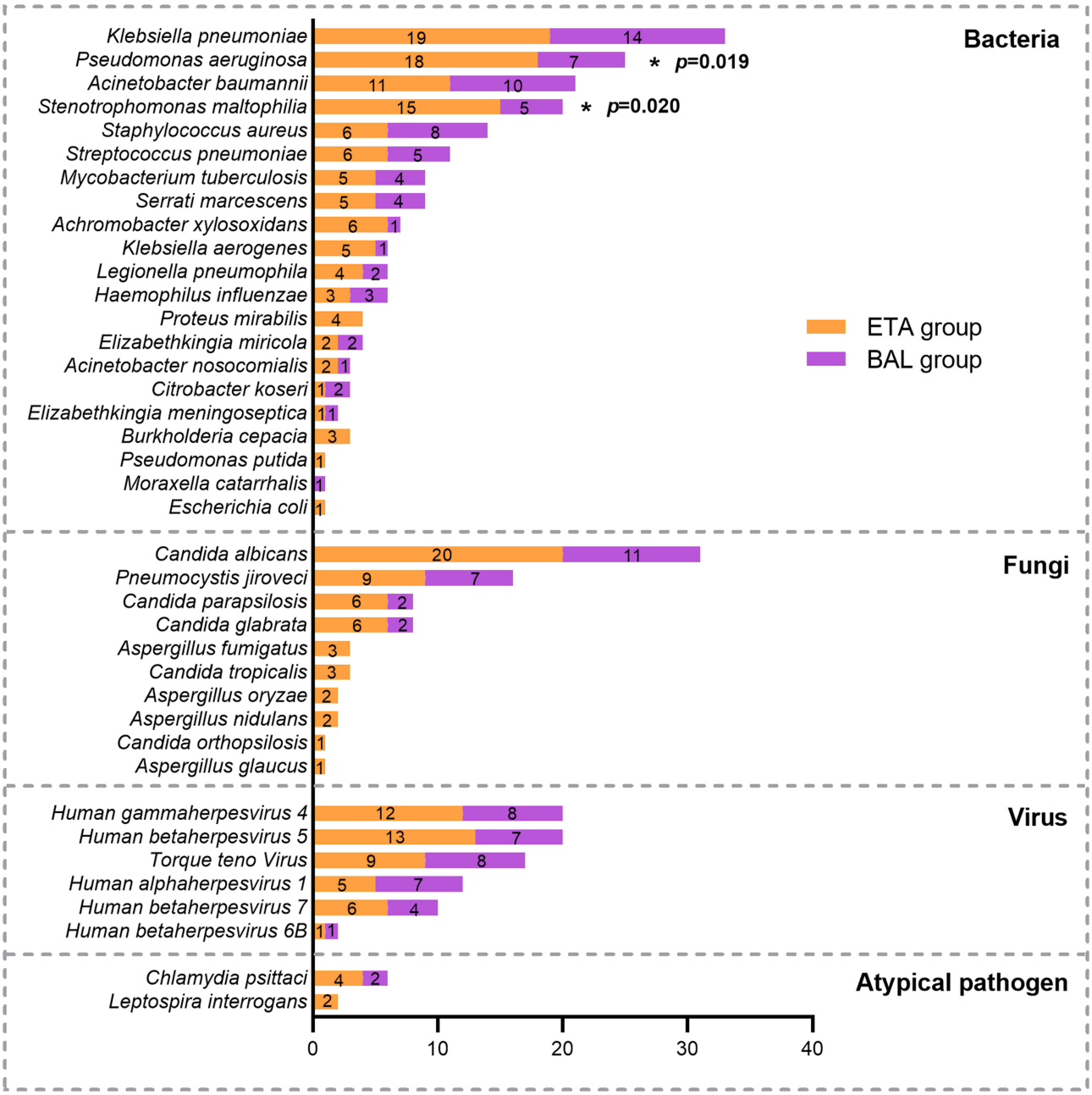

Figure 1 Species distribution of bacteria and fungi and other pathogens detected by mNGS.
Notes: *$p < 0.05$.

In the ETA group, 25 cases (41.7%) had no changes and continued the empirical treatment, 18 cases (30.0%) were adjusted with antibiotics, 10 cases were adjusted with antifungal or antiviral agents, and 7 cases were treated with de-escalation. Among the 57 cases of BAL group, 30 cases (52.6%) continued to empirical treatment without change, which was slightly higher than that of ETA group, 10 cases (22.8%) received targeted therapy, and 12 cases (21.1%) were adjusted with antifungal or antiviral agents (Figure 4).

The diagnostic performance of mNGS results was shown in Table 3. Compared with the BAL group, mNGS of ETA group showed the highest sensitivity 98.1% (95 % CI, 94.6%–100.0%) ($p = 0.008$). The positive predictive value (PPV) 91.4% (95% CI, 84.2%–98.6%) and negative predictive value (NPV) 50.0% (95% CI,19.3%–100.0%) were higher than that in the BAL group.

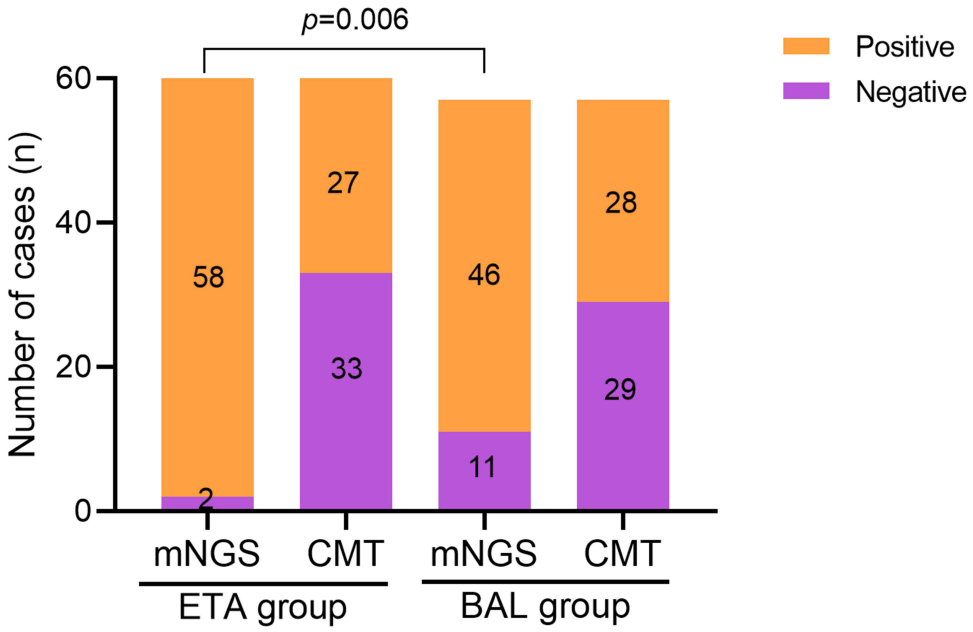

Figure 2 Positivity rate comparison between mNGS and CMT.

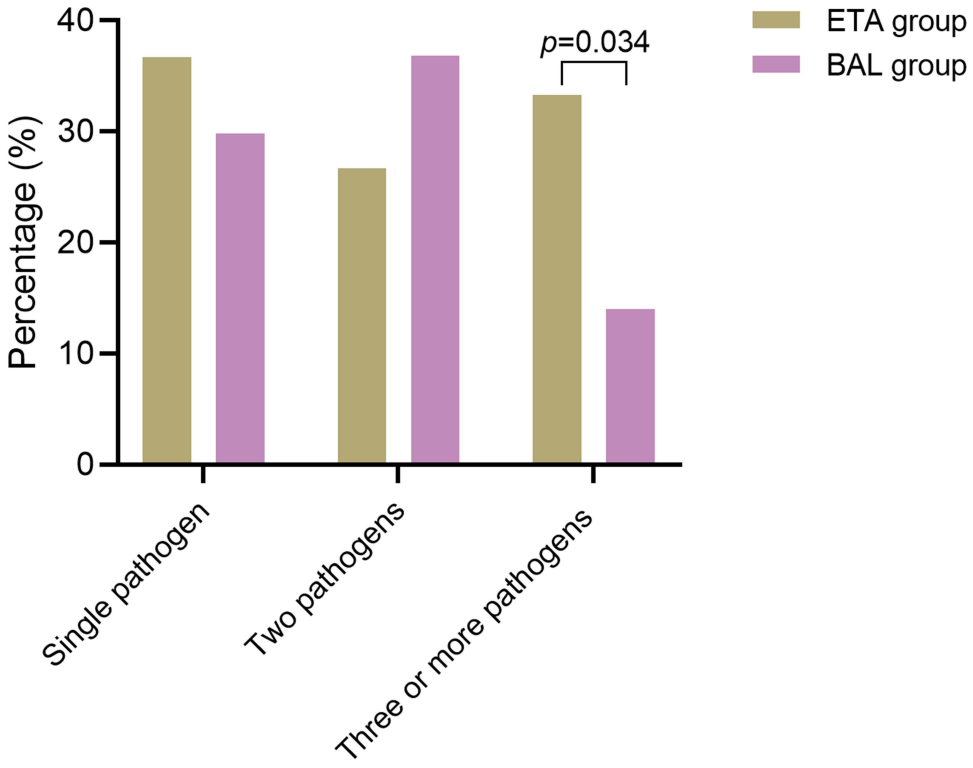

Figure 3 Types of pathogenic microorganisms detected by mNGS.

The clinical outcome of patients with severe pneumonia was shown in Table 4. More frequent development of severe complications, including septic shock (46.7% vs 22.8%, $p = 0.007$) and acute kidney injury (26.7% vs 7.0%, $p = 0.005$) were found in the ETA group. Despite active treatment, the overall mortality was 46.1%, and there was no significant

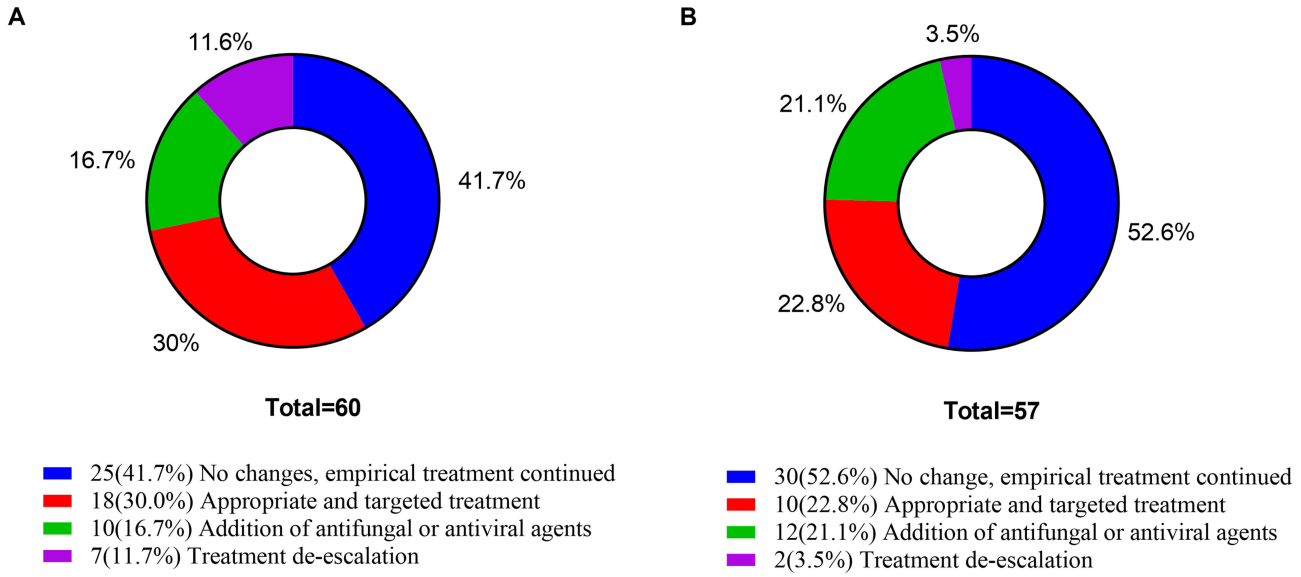

Figure 4 Clinical Impact of mNGS positive results. (**A**). ETA group; (**B**). BAL group.

difference in mortality between the two groups. Compared with the BAL group, the proportion of CRRT and mechanical ventilation in the ETA group was higher. However, there was no significant difference in the hospital length of stay and duration of mechanical ventilation between both groups ($p > 0.05$).

Table 3 Diagnostic Performance of mNGS Results

	Group	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
mNGS	ETA	98.1(94.6–100.0)	16.7(13.2–46.5)	91.4(84.2–98.6)	50.0(19.3–100.0)
	BAL	83.3(72.8–93.9)	33.3(2.5–64.1)	87.0(77.2–96.7)	27.3(0.9–53.6)
p value		**0.008**	0.475	0.466	0.538

Note: Bold type indicates statistical significance ($p < 0.05$).

Table 4 Clinical Outcomes of Patients with Severe Pneumonia

Clinical outcomes	ETA group (n = 60)	BAL group (n = 57)	*p* value
Complications, n(%)			
Sepsis shock	28(46.7)	13(22.8)	**0.007**
Acute kidney injury	16(26.7)	4(7.0)	**0.005**
Mortality, n(%)	28(46.7)	26(45.6)	0.909
CRRT, n(%)	11(18.3)	1(1.8)	**0.003**
ECMO, n(%)	4(6.7)	3(5.3)	1.000
Mechanical ventilation, n(%)	57(95.0)	44(77.2)	**0.005**
Mechanical ventilation time, median (IQR) (hours)	409.0(192.8–562.0)	289.0(119.5–364.8)	0.288
Hospital length of stay, median (IQR) (days)	29.5(18.5–43.0)	18.5(12.5–30.5)	0.082

Note: Bold type indicates statistical significance ($p < 0.05$).
Abbreviation: CRRT Continuous Renal Replacement therapy, ECMO Extracorporeal Membrane Oxygenation.

Discussion

Patients with severe pneumonia are at increased risk of mortality and resource consumption compared to those without the disease, as they require more prolonged hospital stays and intensive care unit treatment.[12] It is very important to identify potential pathogens for appropriate and effective treatment and disease surveillance of affected individuals. Common pathogens of pulmonary infection include bacteria, fungi and viruses. Previous studies have documented that quantitative cultures from BAL or specimens collected through protected brush catheters offered improved diagnostic information in patients with pneumonia.[13] However, conventional culture methods were limited to fungi and bacteria due to their time-consuming and low sensitivity. In recent years, mNGS has emerged as a promising new technique for the detection of pathogenic microorganisms. Although many studies have evaluated the performance of BAL mNGS in various patient populations,[14] the diagnostic value of ETA and BAL mNGS in severe pneumonia has not been thoroughly investigated. Therefore, the objective of this study was to evaluate the diagnostic utility of ETA and BAL mNGS in severe pneumonia.

Our mNGS findings showed that the positivity rates of ETA and BAL pathogen detection were 96.7% and 80.7%, respectively, whereas the positivity rate of ETA pathogen detection was significantly higher than that of the BAL group, and significantly higher than the positivity rate of CMT in both groups. The low positivity rate of CMT may be due to limitations in culture conditions and the use of antimicrobial drugs. Research evaluating the efficacy of BAL and ETA in diagnosing bacterial infections has reached several conclusions. Some studies have found BAL quantitative culture to have no significant diagnostic advantage over ETA.[15] Nan et al reported that there was no significant difference in the positive rates of pathogen detection by routine endotracheal suctioning + mNGS and BALF + mNGS ($p > 0.05$).[16] Another study found that the consistency of mNGS between sputum and BAL in children with lower respiratory tract infection was poor, with sputum virus detection being more reliable than BAL.[17]

In our study, we found that among the bacteria detected, *K. pneumoniae* was the most common microorganism, followed by *P. aeruginosa*. The positive rates of *P. aeruginosa* and *S. maltophilia* in ETA group were significantly higher than those in the BAL group. These two bacteria are commonly associated with hospital-acquired pneumonia. For patients with tracheal intubation or tracheostomy, some bacteria, such as *P. aeruginosa, A. baumannii* and *S. maltophilia*, are often colonized in the airway. In terms of mixed infection, the proportion of three or more pathogens in the ETA group was significantly higher than that in the BAL group. ETA was a non-invasive method with simple operation, fast sampling and low cost, but it was prone to contamination by bacteria in the upper respiratory tract. BAL sampling, on the other hand, could avoid this contamination, but it was an invasive method that requires professionals and can be complex. It was not suitable for patients with complex or serious diseases.

This study compared the impact of microbiology-based information on the clinical outcomes related to diagnostic strategies. The study showed that 58.3% of patients in the ETA group and 47.4% in the BAL group received antibiotic adjustment based on the microbiology results. However, patients in ETA group have more serious complications, including septic shock and acute kidney injury. Compared with BAL group, the proportion of CRRT and mechanical ventilation in ETA group was higher. Overall, the study observed similar mortality rates between the two groups. In a randomized clinical trial, based on the results of ETA culture, the management of VAP patients led to similar clinical results in those of quantitative BAL culture, and the 28-day mortality was similar (25.0% vs 37.8%, $p = 0.353$), and there was no difference between the two groups in the duration of mechanical ventilation, antibiotic treatment, secondary complications, VAP recurrence and hospitalization time.[18] These findings were consistent with those reported in other studies, indicating that the use of invasive or non-invasive strategies in quantitative culture did not significantly impact the clinical outcomes.[19,20]

There are several limitations to this study. First, selection bias may have occurred as it was a single-centre study and the high cost of the mNGS limited the sample size. The extensive application of these findings to the majority of patients with severe pneumonia would require a multi-centre prospective study with a large sample size. Second, some of the patients had already been taking antibiotics prior to sampling, and although there was no difference in the proportion of pre-sampling antibiotic use between the two groups, studies that provide information on antibiotic exposure before

sampling. Third, only DNA process was performed, and a small portion of enrolled cases had RNA mNGS. Therefore, our work did not evaluate the clinical utility and impact of RNA tests.

Conclusion

In conclusion, we found that the varying methods of respiratory specimen sampling led to diverse microbiological results in individuals enduring severe pneumonia. Moreover, we found that although the combination of ETA and BAL with mNGS was beneficial for the pathogenetic diagnosis of these patients, but ETA was more suitable for patients with critical illnesses. It is suggested that the sampling method should be determined by the combination of clinical symptoms and the patient's own condition.

Data Sharing Statement

The datasets generated and analyzed during the current study are not publicly available due to privacy or ethical restrictions but are available from the corresponding author on reasonable request.

Ethics Statement

The Institutional Medical Ethics Committee of Taizhou Hospital of Zhejiang Province granted approval for this retrospective study. Written informed consent was obtained from each patient, and identifying information was removed.

Author Contributions

Peng Zhou and Dehua Zhang contributed equally to this work and shared the first authorship. All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing interests.

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