Research Paper

Application of Alveolar Lavage Fluid Second-Generation Sequencing in the Treatment of Severe Pneumonia

MIN HE, YALIN CHEN AND LONG YU[1]*

Department of Respiratory and Critical Care Medicine, Renmin Hospital, Hubei University of Medicine, [1]Emergency Department, Shiyan Maternal and Child Health Care Hospital, Shiyan, Hubei 442000, P. R. China

He *et al.*: Treatment of Severe Pneumonia with Unidentified Pathogenic Bacteria

To observe the application of alveolar lavage fluid second-generation sequencing in diagnosing and guiding the treatment of patients with severe pneumonia with unidentified pathogenic bacteria and to provide novel ideas and methods for the effective clinical treatment of this disease. The clinical data of 80 patients with severe pneumonia included in our intensive care unit from June 2020 to June 2022 were analyzed and all patients had undergone metagenomic next-generation sequencing and traditional cultures (alveolar lavage fluid culture, sputum culture) to analyze the advantages of metagenomic next-generation sequencing in detecting pathogens in patients with severe pneumonia. The positive rate of metagenomic next-generation sequencing pathogen detection was higher than that of conventional culture. In patients who were negative for conventional culture and positive for metagenomic next-generation sequencing, metagenomic next-generation sequencing was able to further identify multiple pathogenic infections. In terms of pathogen distribution, metagenomic next-generation sequencing detected 71 bacterial, 39 fungal and 3 viral strains. In mixed infections, metagenomic next-generation sequencing yielded a higher rate of positive diagnosis. In addition, metagenomic next-generation sequencing had a higher pathogen detection rate in patients with combined underlying diseases and metagenomic next-generation sequencing could identify specific pathogenic infections especially in patients with combined immunocompromised conditions. Metagenomic next-generation sequencing could improve the detection rate of pathogenic microorganisms in patients with severe pneumonia and could be used as a complementary test for patients negative for conventional cultures. Metagenomic next-generation sequencing has advantages in the diagnosis of mixed infections and can identify multiple pathogenic infections simultaneously. Clinically, early application of metagenomic next-generation sequencing in patients with severe pneumonia is recommended for increased clinical benefit.

Key words: Macro genomic next-generation sequencing, pneumonia, alveolar lavage fluid, pathogen, bacterial infections

Severe Pneumonia (SP) is a common respiratory critical disease in Intensive Care Unit (ICU), and its pathogens are mainly bacteria, as well as fungi, viruses and atypical pathogenic bacteria, etc. Its clinical symptoms are mostly fever, cough, sputum, shortness of breath, dyspnea and the course of the disease is mostly rapidly progressing, which can result in Acute Respiratory Distress Syndrome (ARDS), sepsis and eventually Multi-Organ Dysfunction Syndrome (MODS), with difficulty in treatment and high morbidity and mortality[1]. Although new modern medical technologies are constantly developing and improving and new anti-infective drugs are emerging, which are effective in SP caused by common bacterial infections, but at the same time, multi-drug resistant bacteria are increasingly available and the increase of infection with multi-drug resistant bacteria is continuously raising the difficulty of treatment, so the mortality rate of SP remains high[2]. It has been reported that in recent years, with the increase in per capita life expectancy, the development of medicine, the increased frequency of antimicrobial drugs and the irregular use of antibiotics, the emergence of multi-drug resistant bacteria has increased in proportion, enhancing the difficulty of treatment, so the mortality rate is at the top[3]. It has also been shown that SP currently ranks first among the causes of death from infectious diseases worldwide[4].

The treatment methods commonly used in SP clinics

***Address for correspondence**
E-mail: xiyuan93429@163.com

include anti-infective therapy, anti-inflammatory therapy, immunomodulatory therapy and organ function support therapy and organ function support therapy includes mechanical ventilation, nutritional support, hemofiltration purification and Extracorporeal Membrane Oxygenation (ECMO), among which mechanical ventilation is an important treatment tool[5]. At present, most tertiary hospitals in China are equipped with the above organ function support therapy in terms of hardware and equipment, but it is still necessary to optimize the treatment plan, including the standardized use of anti-infective drugs, selection of appropriate timing of organ function support intervention and timely adjustment of treatment strategy according to the condition, in order to better improve the success rate of resuscitation treatment[6]. Despite the fact that many studies on SP have been conducted, most of them are based on Western medicine and not many of them are combined with Chinese medicine. In the actual clinical practice of our department, in addition to the basic Western medical treatment, a growing number of Chinese medical treatments have been added to the treatment of SP and the combination of Chinese and Western medicine can deliver favorable outcomes; therefore, the combination of Chinese and Western medicine in the treatment of SP is worthy of further study[7]. SP can be classified as "lung distension" and "asthma" in Chinese medicine. Elderly people are susceptible to external invasion due to deficiency of vital energy, deficiency of the body and deficiency of lung health and thus are susceptible to invasion of external evil, which invades the lungs and turns heat into heat, which injures lung fluid and refines liquid into phlegm. This leads to the onset of the disease. The common type of evidence in elderly patients with SP is phlegm-heat congestion of the lung. Although there are a number of treatments available for SP, earlier tests are particularly important due to its severity[8,9].

Traditional pathogenic diagnostic methods, such as pharyngeal swabs, sputum cultures, serum pathogen antibody tests and blood cultures, fail to meet the demand for rapid and precise clinical treatment. With the development and improvement of medical level, the fiberoptic bronchoscopy technology has been increasingly applicable in respiratory diseases, as it allows direct access to the site where the lesion is located and operation under visualization, which can rapidly control infection, relieve the patient's condition, improve clinical symptoms and restore pulmonary ventilation as soon as possible[10]. Fibro bronchoscopy possesses both therapeutic and diagnostic effects, allowing direct microscopic observation of the lesion, parallel Bronchoalveolar Lavage (BAL) and aspiration of BAL Fluid (BALF) for pathogen detection and BAL *via* fibronectomy with a wide sampling range to reach the distal lung parenchyma and collection of BALF specimens from a broad range of lung parenchyma for pathogen detection, which can truly reflect the patient's pneumonia pathogenesis and improve the diagnosis rate of respiratory pathogens[11,12]. Alveolar lavage has become an independent treatment for pulmonary diseases with the advantages of easy operation, safety and reliability, and less pain.

Next-Generation Sequencing (NGS), i.e., high-throughput sequencing, is a novel sequencing method compared with the first-generation Deoxyribonucleic Acid (DNA) sequencing technology, offering the advantages of high throughput, low cost, high sensitivity and simultaneous determination of all nucleic acid sequences in a sample, which enables a dramatic improvement in sequencing speed and sequencing cost[13]. With the reduction of NGS sequencing cost and the increase of sequencing data volume, NGS has been rapidly developed, making it widely used in various fields such as detection and typing of pathogenic microorganisms of infectious diseases and drug resistance gene detection, in addition to it could avail genomic information required for pathogen evolutionary information tracking, strain identification and drug resistance detection[14].

Metagenomic NGS (mNGS) provides timely and accurate pathogenic detection and it plays an essential role in guiding clinical diagnosis and treatment. Presently, no uniformity is available for the interpretation of mNGS reports and the prediction of the diagnostic value of SP varies. In view of the epidemiological, etiological, clinical symptoms and new features of treatment of this disease, this study will focus on clinical research for SP. To explore the value of alveolar lavage fluid second-generation sequencing for the pathogenic diagnosis of SP, patients with a preliminary clinical diagnosis of SP comprised the study population, aiming to identify the main pathogenic types and clinical features of SP in the region and to provide fresh ideas for the clinical diagnosis and treatment of lobar pneumonia

in children.

MATERIALS AND METHODS

General data:

Eighty patients with SP were selected from our ICU from June 2020 to June 2022. Statistical Package for the Social Sciences (SPSS) 23.0 software was adopted, a random number table was generated and the patients were randomly divided into two groups. Prior to enrollment, the study was conducted with informed consent and signed by the patients. The study protocol was approved by the hospital ethics committee and all processes complied with the Declaration of Helsinki Ethical Guidelines for clinical research. Clinical data were collected on these patients, including gender, age, underlying disease, imaging presentation, immunosuppression, immune status, alveolar lavage fluid routine, smear, 1,3-beta-D Glucan/Galactomannan assay (G/GM test) and culture results, mNGS results and final outcome and all patients who deteriorated and were automatically discharged were considered dead. Informed permission was obtained from patients and informed consent was signed prior to enrollment in this study. The study protocol was approved by the hospital ethics committee and all processes complied with the Declaration of Helsinki ethical guidelines for clinical research as shown in Table 1.

Diagnostic criteria:

All patients met the clinical diagnostic criteria for SP in the 2019 Guidelines for the Treatment of Community-Acquired Pneumonia in Adults[15] developed by the American Thoracic Society (ATS) and Infectious Diseases Society of America (IDSA). The diagnostic criteria for SP were as follows:

Two primary criteria: Infectious shock requiring the application of vasoactive drugs and respiratory failure demanding mechanical ventilation.

Nine secondary criteria: Respiratory rate ≥30 breaths/min; Arterial Oxygen Partial Pressure/Fractional inspired Oxygen (PaO_2/FiO_2) ≤250 mmHg; multilobar exudative lesions; mental confusion/disorientation; elevated Blood Urea Nitrogen (BUN) ≥20 mg/dL); leukopenia (White Blood Cells (WBC) $<4\times10^9$/L); thrombocytopenia (Platelet Count (PLT) $<100\times10^9$/L); hypothermia (central body temperature <36°); hypotension requiring fluid resuscitation. SP was determined by meeting one of the primary criteria or meeting three or more of the secondary criteria.

TABLE 1: COMPARISON OF GENERAL DATA

n	80
Male/female	51/29
Age	69.2±4.31
Length of hospitalization	20.3±3.46
Clinical features	
Leukocytes [10^9/l]	15.62±8.21
Calcitoninogen [ng/ml]	0.6±0.21
Antibiotics used before mNGS	56
Hormone use	22
Underlying disease [cases (%)]	
COPD	5
Cardiovascular disease	35
Stroke	12
Diabetes mellitus	17
Chronic kidney disease	1
Tumor	10
Immunosuppression	5
Tracheal intubation	37
Prognosis [cases (%)]	
Improvement	52
Progression	12
Death	16

Inclusion and exclusion criteria:

Inclusion criteria: Patients confirmed by pulmonary imaging and laboratory examination; patients manifested with cough, fever, sputum, chest pain, chills, dyspnea and accelerated respiratory rate; patients with APACHE II score ≥20; patients who had informed consent and patients without large pleural effusion in the lungs.

Exclusion criteria: Patients complicated with malignancy; patients with combined bronchial foreign bodies; patients with contraindications to fibrinoscopy or without tolerance; patients with systemic infection; patients with serious complications of other organs; patients with admission time <48 h; patients who cannot receive BAL for various reasons and patients with combined mental abnormalities.

Research methods:

In the study group, pathogens were detected by traditional methods and next-generation gene sequencing of alveolar lavage fluid, while in the control group pathogens was detected only using traditional methods. Specifically, alveolar lavage fluid was collected after bronchoscopy in both groups and sent for general bacterial culture, *Mycobacterium tuberculosis*, fungi and bacterial smear and the study group sent for alveolar lavage fluid mNGS on this basis.

BAL method: Patients fasted for 6 h before the procedure, Olympus BF260 electronic bronchoscope was used and intravenous sedation plus local anesthesia was administered. All study subjects were given informed consent by their guardians before fiberoptic bronchoscopic alveolar lavage was performed. Local surface anesthesia with sedation was used and oxygen, Electrocardiogram (ECG) and oxygen saturation were routinely administered by mask during the operation. The child was placed in the supine position and the fiberoptic bronchoscope was inserted through the nose, usually on the healthy side first and then on the affected side, with the focus on the areas where physical examination or imaging suggested abnormalities. BALF was obtained by post-negative pressure suction, and the specimen was placed in a disposable sterile siliconized collector for examination. The BALF specimen was partially tested immediately after collection and partially placed in an insulated box with an ice pack and placed in a -80° refrigerator for freezing pending examination. The fiberoptic bronchoscope was slowly withdrawn. During the operation, oxygen was administered, oxygen saturation, blood pressure and heart rate were routinely monitored and the patient's breathing, lip color and facial color were closely observed. The lavage solution was 37° saline, given at 5 ml/time and the lung segments with heavy inflammation were lavaged at least 3 times, with lavage recovery ≥2 ml indicating adequate lavage and the total amount of lavage solution was 5-10 ml/kg. BAL was performed in the heavily inflamed areas and if the inflammation was extensive, the left lingual branch and right middle lobe were selected for lavage.

Alveolar lavage fluid mNGS detection method in the study group: BALF was delivered to Shenzhen Huada Gene Co., Ltd. for nucleic acid extraction followed by PMseq pathogenic microbial DNA high-throughput sequencing and the sequenced pathogenic sequences were compared with the pathogen library to obtain the final results. The criteria for reporting pathogenic bacteria were referred to the existing literature[6].

In addition, clinical data such as the febrile course (onset to preadmission) and the site of pneumonia lesions in imaging examinations were collected from both groups. Indexes such as Erythrocyte Sedimentation Rate (ESR), C-Reactive Protein (CRP), Calcitoninogen (PCT), coagulation function, mycoplasma pneumoniae antibody and virus antibody tests were collected from both groups.

Result analysis:

The criteria for determination of positive mNGS results were as follows; the relative abundance of pathogenic genera identified by mNGS was required to be >30 %. When the same pathogenic bacteria were detected in mNGS and conventional culture, the number of specific sequences (read number) should be >50. When microorganisms were identified only by mNGS, they were considered as new potential pathogens. In either mNGS or conventional culture, when microorganisms are detected and the above criteria are met, at least two clinicians are required to differentiate colonization, contamination and infection in the context of the clinical background.

Statistical methods:

Data were analyzed using SPSS 23.0 software. $p<0.05$ was considered as statistically significant and all tests were two-sided. Count data were expressed as number of cases and comparative analysis was performed by Pearson test. Fisher's exact test was performed for comparative analysis. The measurement data were presented as mean±standard deviation and tested for

conformity to a normal distribution, and for data that did not conform to a normal distribution, nonparametric tests were performed.

RESULTS AND DISCUSSION

In the alveolar lavage fluid of 80 patients with SP, mNGS was positive in 78 cases, with a positivity rate of 97.5%. Conventional culture detected 51 positive cases, with a positivity rate of 63.75 % ($p<0.001$) as shown in Table 2.

In this study, 26 cases were negative for conventional culture and positive for mNGS and among these specimens, the number of pathogens detected was further analyzed. Among them, one pathogen was detected in 10 cases, two pathogens were detected in 12 cases and three pathogens were detected in four cases.

A total of 113 microorganisms (71 bacteria, 39 fungi and 3 viruses) were detected by mNGS. Among the bacteria, the genus *Fusobacterium* (16/71) was the most abundant, followed by *Streptococcus* (12/71), *Klebsiella* (11/71) and *Pseudomonas* (6/71). Among the fungi, *Candida* spp. (30/39) was the most common. Three viruses were detected in the specimen, including coronavirus (2 strains) and *Cytomegalovirus* (1 strain). Fifty microorganisms (34 bacterial and 16 fungal) were detected by conventional culture and the most detected bacteria were *Fusarium* spp. (13/34) and the most detected fungi were also *Candida* spp. (12/16) and no viruses were detected in the specimensas shown in Table 3.

TABLE 2: DETECTION OF PATHOGENS IN EACH AGE GROUP (x±s)

Group	Detected	Undetected	χ^2/t	p
mNGS	78	2	5.4	<0.001
Conventional culture	51	29	0.06	0.748

TABLE 3: DISTRIBUTION OF PATHOGEN GENERA DETECTED BY mNGS AND CONVENTIONAL CULTURE

Pathogen genus	mNGS detected (strain)	Detected by conventional culture (strain)
Bacteria	71	34
Bacillus spp.	16	13
Streptococcus spp.	12	0
Klebsiella spp.	11	10
Pseudomonas spp.	6	3
Enterococcus spp.	5	0
Enterobacter spp.	5	4
Staphylococcus spp.	5	1
Chromobacterium spp.	2	1
Narrow-feeding *Aeromonas* spp.	2	2
Burkholderia spp.	2	0
Micrococcus spp.	2	0
Corynebacterium striatum	1	0
Fungi	39	16
Candida spp.	30	12
Aspergillus spp.	6	4
Pneumocystis spp.	2	0
Cryptococcus spp.	1	0
Viruses	3	0
Coronavirus	2	0
Cytomegalovirus	1	0

With 80 cases of SP, single pathogen infections (1 pathogen detected) were detected in 22 cases by mNGS, compared with 35 cases by the conventional method, which was higher than mNGS. There was a statistically significant difference between the two methods only in terms of single fungal infections. In terms of mixed infections (two or more pathogens detected), mNGS detected 47 positive specimens, which was markedly higher than culture (9 cases) and there was a significant difference between the two methods ($p<0.001$). Among the types of infection, mixed bacterial and fungal infections were the most common in both methods, followed by mixed bacterial and bacterial infections. In addition, mNGS detected one case of mixed bacterial+fungal+viral infection as shown in Table 4.

In the study, 62 patients had comorbid underlying diseases and in these patients, mNGS was detected positively in 48 cases and negatively in 1 case. The culture method detected 34 positive cases and 15 negative cases, with statistically significant differences in the rate of positive pathogens detected between the two methods ($p<0.001$). Meanwhile, in 21 patients without underlying disease, there was a statistically significant difference between mNGS and conventional culture ($p<0.001$) as shown in Table 5.

SP is a respiratory disease with a high mortality rate, usually with an acute onset and rapid progression, which is mainly attributed to bacterial and viral attacks on the lungs and seriously threatens the respiratory function of the patient, resulting in respiratory failure if there is no timely intervention, seriously threatening the physical and mental health and life safety of the patient[16]. In the past, mechanical ventilation and anti-infective treatment were mainly used in clinical practice, but it was hardly possible to remove the inflammatory secretions (residual in the respiratory airway) completely and the treatment was not effective[17]. With the development of fiberoptic bronchoscopy, secretions in the respiratory airway can be removed under direct vision and antibiotics can be injected directly into the lesion area, which can fight infection to a certain extent. BAL is known as "liquid lung biopsy", directly taken from the lesion, which may be less contaminated and more reflective of pulmonary pathogenesis than sputum culture and thus it can provide an accurate pathogenic diagnosis for treatment and provide a basis for antibiotic selection[18]. BALF is currently considered as a more sensitive and reliable method for the diagnosis of pneumonia pathogens. The detection of pathogens in BALF by Fluorescent Quantitative Polymerase Chain Reaction (FQ-PCR) can rapidly and effectively improve the pathogen detection rate[19]. Early and rapid accurate determination of pathogenic microorganisms and targeted use of antibiotic therapy are of great importance for effective preventive control, clinical diagnosis and treatment and improved prognosis.

TABLE 4: COMPARISON OF PATHOGENIC INFECTION TYPES

Pathogen detection	mNGS	Conventional culture	χ^2	p
Single pathogen infection	22	35	7.37	0.025
Bacteria only	17	23	2.15	0.262
Fungus only	3	12	3.21	0.014
Virus only	2	0	5.29	0.476
Mixed infections	47	9	5.267	<0.001
Bacteria+bacteria	6	4	1.38	0.512
Bacteria+fungi	40	5	4.63	<0.001
Bacteria+virus	0	0	0.34	-
Bacteria+fungus+virus	1	0	2.02	1

TABLE 5: COMPARISON BETWEEN mNGS AND CONVENTIONAL CULTURE

Group	Total number	mNGS		Conventional culture		p
		Positive	Negative	Positive	Negative	
With underlying disease	62	60	2	39	23	<0.001
No underlying disease	18	18	0	8	10	<0.001

Traditional microbial culture techniques are characterized by low positive rates and long culture cycles, while immunological methods such as antigen/antibody and pathogen-specific nucleic acid sequence PCR methods target only known pathogens and fail to detect unknown pathogens[20].

Currently, the traditional pathogenic tests commonly used in clinical practice, such as culture, microscopic examination, serological tests; usually present time-consuming, low positivity rate and other concerns. Low detection rate, in case of interference from anti-infective drugs, specimen sampling, special pathogens and other factors, the positive rate will be even lower, so the sensitivity of detection is unfavorable; small range of detection, culture or molecular biology techniques are only for the detection of a small number of microorganisms, resulting in the prevalence of missed diagnosis, so the specificity of detection is not high; poor timeliness of testing, such as long bacterial culture cycle and pathogenic antibody generation time, resulting in bacterial culture and serological antibody testing cannot diagnose the presenting infection in a timely manner; the lack of reference or basis for identification of rare pathogens, resulting in the failure to identify and diagnose rare pathogens; some test specimens are difficult to obtain or at high risk, such as the difficulty in obtaining material for pathological examination of lung tissues and traditional techniques such as microbial culture and serological testing are not effective enough to achieve high-throughput and large-scale clinical sample detection[21,22]. Therefore, the exploration of new technologies for microbial detection and their application to clinical diagnosis of pathogenesis is a major issue in the field of anti-infection.

NGS technologies, also known as high-throughput sequencing, can allow hundreds of millions of gene fragments to be sequenced simultaneously or independently. Currently, second-generation sequencing technologies commonly used for clinical infectious disease diagnosis include targeted amplicon sequencing and macro genomic sequencing, among which targeted amplicon sequencing, represented by 16S ribosomal Ribonucleic Acid (1 6SrRNA), can only detect specific microorganisms and requires advance knowledge of the pathogenic microorganism type, which does not allow for a comprehensive assessment of clinical infectious disease etiology[10]. In contrast, mNGS technology can obtain the gene sequences of different pathogenic microorganisms for identification without relying on predicted pathogens, including DNA sequencing and RNA sequencing (DNAseq and RNAseq), which can identify bacteria, fungi, viruses and parasites simultaneously and even rare and newly discovered pathogenic microorganisms, a feature that is theoretically useful for clinical anti-infection field with some guidance[23].

In this study, mNGS detected 71 bacteria, 39 fungi and 3 viruses. Compared with culture (34 bacteria and 16 fungi), mNGS detected a larger number of pathogens and covered a wider range of microorganisms. It has been suggested that mNGS has advantages in virus detection and can be used as a complementary tool to conventional methods. In this study, three strains of viruses (two coronavirus strains and one *Cytomegalovirus* strain) were detected. One coronavirus strain was detected in a patient with Coronavirus Disease-19 (COVID-19), another human coronavirus strain NL63 was detected in a patient with left lung malignancy after long-term chemotherapy and the *Cytomegalovirus* strain was detected in a patient with SP combined with Human Immunodeficiency Virus (HIV), although all three strains were identified only by mNGS, which may be caused by insufficient sample size, we will consider expanding the sample size for further exploration in the future. *Streptococcus pneumonia* has been the most common causative agent not only for community-acquired pneumonia but also for SP.

In recent years, gram-negative bacilli have assumed an increasingly larger proportion of severe infections[24]. In the present study, both mNGS and culture methods indicated that gram-negative bacilli were the main causative organisms responsible for SP. However, the most common bacteria and fungi detected by both methods in the study were *Acinetobacter baumannii* (16 strains by mNGS and 13 by culture method) and *Candida albicans* (30 strains by mNGS and 12 by culture), which when considered in conjunction with the source of the patient's sample, the clinician needs to integrate the patient's specific situation to distinguish among colonization, contamination or infection. Patients with comorbid underlying disease often have a higher risk of infection. In this study, mNGS also showed a higher detection rate of pathogenic microorganisms in patients with co-morbidities compared to culture, allowing clinicians to respond more quickly and adjust anti-infection regimens. Five patients in the study were immunodeficient and mNGS detected specific pathogens in two cases (*Pneumocystis* in one case and *Cytomegalovirus* in one case). mNGS

detected a mixed infection of bacteria+fungus+virus in one culture-negative patient. All three patients were discharged after adjustments to the treatment plan based on the mNGS results. mNGS has obvious advantages in the diagnosis of pathogenic microorganisms in this special population.

Some deficiencies were identified in this study, firstly, due to the limitations of objective factors such as research funding and research time, there were inevitably deficiencies in this study such as small sample size and single study center. The geographical location of the patients was also limited and no multicenter joint study was conducted. In addition, social factors such as family economic status and education level were also related to the results, so some selection bias existed; second, the sample size of this study was small, with only 18 cases, which may leave some shortcomings in representativeness; third, our results came from one sequencing company and there may be some differences in the selection of macro genetic libraries, etc.; finally, no uniform standard was available to interpret the macro-sequencing results and the distinction between colonization and infection remains unclear and this article determines whether the pathogen is pathogenic based on the history and physical examination by the patient's primary care physician, which may be subjective.

In this study, we found that viral, fungal, atypical pathogens and bacterial co-infections were the most common pathogens in patients with SP. In terms of pathogenic microorganism detection positivity, mNGS is superior to conventional culture and can be used as a complementary test for patients with SP negative for conventional culture. mNGS is superior in the diagnosis of mixed infections and can identify multiple pathogenic infections simultaneously. Clinically, early application of mNGS in patients with SP with combined immunodeficiency is recommended for increased clinical benefit.

Author's contributions:

Min He and Yalin Chen have contributed equally to this work.

Conflict of interests:

The authors declared no conflict of interests.

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