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Improving the clinical effectiveness of metagenomic next generation sequencing (mNGS) in infection disease diagnosis and treatment: Linking the NGS specialists and clinicians,
volume II

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Published in
Frontiers in Cellular and Infection Microbiology

ISSN 1664-8714
ISBN 978-2-8325-4175-3
DOI 10.3389/978-2-8325-4175-3

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Improving the clinical effectiveness of metagenomic next generation sequencing (mNGS) in infection disease diagnosis and treatment: Linking the NGS specialists and clinicians, volume II

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Citation

Ang, L., Zhou, X., Zheng, B., eds. (2023). *Improving the clinical effectiveness of metagenomic next generation sequencing (mNGS) in infection disease diagnosis and treatment: Linking the NGS specialists and clinicians, volume II*.

Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-4175-3

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 27 October 2022
DOI 10.3389/fcimb.2022.1026739

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 24 August 2022
ACCEPTED 10 October 2022
PUBLISHED 27 October 2022

CITATION
Zhao M, Yue R, Wu X, Gao Z, He M and Pan L (2022) The diagnostic value of metagenomic next-generation sequencing for identifying *Pneumocystis jirovecii* infection in non-HIV immunocompromised patients. *Front. Cell. Infect. Microbiol.* 12:1026739. doi: 10.3389/fcimb.2022.1026739

The diagnostic value of metagenomic next-generation sequencing for identifying *Pneumocystis jirovecii* infection in non-HIV immunocompromised patients

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Background: *Pneumocystis jirovecii* pneumonia (PJP) remains an important cause of morbidity and mortality in non-HIV immunocompromised patients especially in transplant recipients. But its diagnosis remains challenging due to the insufficient performance of conventional methods for diagnosing *Pneumocystis jirovecii(P. jirovecii)* infection. Therefore, the auxiliary diagnostic function of metagenomics next-generation sequencing (mNGS) in clinical practice is worth of exploring.

Method: 34 non-HIV immunocompromised patients who were diagnosed as PJP by clinical manifestations, imaging findings, immune status of the host, and Methenamine silver staining were tested by mNGS from October 2018 to December 2020 in Sichuan Provincial People's Hospital. The clinical performances of mNGS for *P. jirovecii* infection diagnosis were also evaluated with genome reads abundance and comparing with other traditional diagnostic methods.

Results: We diagnosed a total of 34 non-HIV PJP patients by the clinical composite diagnosis. Our data shows that, compared with the clinical microbiological test, the detection rate of mNGS for *P. jirovecii* in non-HIV infected PJP patients is significantly higher than that of Methenamine silver staining and serum 1-3-β-D-glucan. mNGS can be used as an auxiliary diagnostic tool to help diagnosis. The number of reads mapped to the genome of *P. jirovecii* and the duration of patients from onset to sampling collection were statistically significant between the two groups (Reads>100 and Reads ≤ 100) (8days *vs*. 23days, p=0.020). In addition, univariate analysis showed that C-reactive protein (15.8mg/L *vs*.79.56mg/L, p=0.016), lactate dehydrogenase (696U/l *vs*. 494U/l, p=0.030) and procalcitonin (0.09ng/ml

vs. 0.59ng/ml, p=0.028) was also statistically significant between the two groups.

Conclusions: An effective detection rate was achieved in PJP patients using mNGS testing of bronchoalveolar lavage fluid (BALF) or blood. The study also confirmed that the abundance of reads of *P. jirovecii* is related to the interval between the onset and sample collection. And the inflammation status during simultaneous mNGS detection might determine the abundance of pathogens. Hence, we conclude that the mNGS strategy could benefit disease diagnosis as well as treatment when complicated clinical infections appeared.

KEYWORDS

metagenomic next-generation sequencing (mNGS), *Pneumocystis jirovecii* (*P. jirovecii*), infection, transplantation, immunocompromised

Introduction

Pneumocystis jirovecii pneumonia (PJP) is a common opportunistic infection in the immunocompromised population (Eddens and Kolls, 2015). PJP cases have been reported frequently in solid organ transplant recipients, particularly in renal transplant recipients (Yiannakis and Boswell, 2016; Chen et al., 2020; Le Gal et al., 2020), and other patients like cancer, patients with congenital or acquired immunodeficiency or patients treated with immunosuppressive drugs, and so on (Wickramasekaran et al., 2017; Chen et al., 2020). With the progress of medical technology, the population of organ transplantation or immunosuppression is gradually increasing, so the population of PJP may also increase. Compared with the human immunodeficiency virus (HIV)-PJP, the early clinical symptoms of non-HIV PJP are atypical and nonspecific, and more likely to lead to alveolar damage and respiratory failure, and rapidly develop into severe pneumonia, resulting in extremely high mortality. (Monnet et al., 2008; Tasaka et al., 2010; Roux et al., 2014). At present, the incidence of PJP in the non-HIV population continues to increase, which deserves attention (Cillóniz et al., 2019).

Rapid pathogen diagnosis and accurate treatment play a key role in improving outcomes in non-HIV patients with PJP (Zhang et al., 2021). Since *P. jirovecii* cannot grow stably *in vitro*, the detection tools for *P.jirovecii*, such as microscopy and Gomori's methenamine silver staining, have limitations with low positive rate. Because these traditional methods require a high pathogen burden in the lungs and experienced microbiologists to ensure microscopic detection rates of *P. jirovecii*, which may lead to a certain false negative (Ma et al., 2018; Le Gal et al., 2020; Jiang et al., 2021). Besides, real-time PCR based diagnostic tests have significantly improved sensitivity and specificity, while commercial kits have not yet been widely used in clinical in China (Summah et al., 2013; Zhang et al., 2021). Metagenomic next-generation sequencing (mNGS) can complement traditional diagnostic methods through high-throughput sequencing, which can directly detect nucleic acids from pathogens in clinical samples, and then analyze nucleic acid sequences through bioinformatics methods. Comparing with traditional methods, mNGS has a higher detection rate of PJP (Wang et al., 2019; Zhang Y et al., 2019; Irinyi et al., 2020; Li et al., 2020). In addition, compared with other traditional diagnostic methods, the turnaround time of mNGS would be accomplished within 48 hours, therefore, mNGS can play an advantage in clinically auxiliary diagnosis (Somasekar et al., 2017; Chiu and Miller, 2019). mNGS could simultaneously identify bacteria, fungi, viruses, and various parasites from clinical samples such as cerebrospinal fluid, plasma, tissue, pleural fluid, etc. in an unbiased, synchronized, and straightforward manner (Yao et al., 2016; Chen et al., 2021). In recent years, the feasibility of mNGS in the pathogen identifications from clinical samples has been confirmed (Miao et al., 2018).There are some kinds of pathogens, such as fungi or viruses are not detectable by conventional culture methods, but can be detected by mNGS accurately and showed a higher positive detection rate (Charpentier et al., 2017; De La Cruz and Silveira, 2017). Therefore, nowadays, the implementation of mNGS becomes a very important detection method for severe or emerging pathogen infection.

To validate our main hypothesis that mNGS would perform better in emerging infection agents identification, in this study, the clinical data, routine biochemical tests, microbial culture, and mNGS results of 34 non-HIV immunocompromised PJP patients admitted to Sichuan Provincial People's Hospital were analyzed to evaluate the application value of mNGS in clinical diagnosis.

Materials and methods

Study population and specimen collection

We included patients who were highly suspicious of PJP from 2018 to 2020 in Sichuan Provincial People's Hospital. At present, the reference standard for diagnosis of PJP is still mainly to find characteristic cysts and trophozoites through staining and microscopic examination of clinical specimens, combined with the immune status of the host, degree of immunosuppression, imaging characteristics, and other biochemical indicators are taken as the auxiliary diagnosis of PJP. In this study, we combined the above mentioned factors as the reference standard for diagnosing PJP. We didn't conduct PCR, because this detection method is not commonly used in most clinical microbiology laboratories in China (Bandt and Monecke, 2007). Patients were eligible for enrollment if they met all the following criteria:1) Immunocompromised, such as hematological malignancies, solid organ transplantation, hematopoietic stem cell transplantation, rheumatic immune system diseases, long-term use of corticosteroids or immunosuppressants, skin system diseases, etc.; 2) Typical clinical manifestations include subacute attacks, progressive dyspnea, accompanied by symptoms such as fever, dry cough, dyspnea and fatigue, and progressive hypoxemia; 3) Highresolution chest CT showed typical diffuse reticular nodules in both lungs, beginning with hilar nodular interstitial infiltration, mainly diffuse ground-glass opacities, with occasional plaques and consolidations; ([Chinese guidelines for diagnosis and treatment of HIV/AIDS (2018)], 2018; Fishman and Gans, 2019; Wang et al., 2019). 34 immunocompromised patients were diagnosed as PJP based on the host clinical status and immune states, clinical features, imaging findings, the results of Methenamine silver staining, the result of mNGS, and the comprehensive judgment of two senior clinicians in the hospital.

The flow diagram of the study is described in Supplemental Figure S1. Clinical data of all confirmed cases were recorded, including demographic characteristics, sample collection, and clinical microbiological test results. After admission, bronchoalveolar lavage fluid (BALF) or blood was sent for mNGS test.

Plasma sample: Sample processing and DNA extraction

The volume of 3-4 mL of whole blood was drawn from patients, placed in the blood collection tube(BD Biosciences, State of New Jersey, USA) and stored at room temperature for 3-5 minutes before plasma separation and centrifuged at 4,000 rpm for 10 min at 4°C(Eppendorf, Hamburg, Germany)within 8 h of collection. Plasma samples were transferred to new sterile tubes(Gene Era Biotech, California, USA).DNA was extracted from 300 uL of plasma using the TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH, Beijing, China) following the manufacturer's operational manual. The extracted DNA specimens were used for the construction of DNA libraries (Long et al., 2016).

Body fluid sample: Sample processing and DNA extraction

1.5-3mL BALF sample from the patient was collected according to standard procedures. 1.5mL microcentrifuge tube(Gene Era Biotech, California, USA) with 0.6mL sample and 1g 0.5mm glass bead were attached to a horizontal platform on a vortex mixer (Thermo, Massachusetts, USA) and agitated vigorously at 2800-3200 rpm for 30 min. 0.3mL sample was separated into a new 1.5mL microcentrifuge tube and DNA was extracted using the TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH) according to the manufacturer's recommendation.

Construction of DNA libraries

Then, the DNA library was constructed by DNA fragmentation, end repair of blunt ends caused by fragmentation, ligation of adapters for amplification and sequencing, and PCR amplification to enrich the target fragments using the MGIEasy Cell-free DNA Library Prep Set (MGI Tech, Shenzhen, China), according to the instructions of the manual. Agilent 2100 was used for quality control of the DNA libraries. Quality qualified libraries were sequenced by the BGISEQ-50/MGISEQ-2000 platform (Jeon et al., 2014).

Sequencing and bioinformatics analysis

High-quality sequencing data were generated by removing low-quality reads, followed by computational subtraction of human host sequences mapped to the human reference genome (Hg19) using Burrows-Wheeler Alignment (Li and Durbin, 2009). The remaining data by removal of low-complexity reads were classified by simultaneously aligning to four Microbial Genome Databases, consisting of bacteria, fungi, viruses, and parasites.

The classification reference databases were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/). Reference sequences contained 4,945 whole genome sequences of virus, 6,350 bacterial genomes or scaffolds, 1064 fungi related to human infection, and 234 parasites associated with human diseases. The Genbank accession number of *P. jirovecii* reference genome sequences using for the mapping is GCA_001477535.1.

Detection methods for methenamine silver staining and serum 1-3-β-D-glucan

BALF and blood were smeared on glass slides. After drying naturally, the samples were stained with silver staining solution (Zhuhai beso Biotechnology Co., Ltd., Shenzhen, China) according to the operation instructions. After waiting 3-5 min, *P. jirovecii* was microscopically examined for cyst structure.

The cell wall components of fungi(1-3-β-D-glucan) were detected according to the instructions of the fungal (1-3)-β-D-glucan detection (chromogenic method) kit (Dana Biotechnology Co., Ltd., Tianjin, China).

Statistical methods

All continuous variables were expressed as medians, the Mann-Whitney U test was used to compare the differences of continuous variables between the two groups, continuous variables with a *P* value 0.05 were considered statistically significant, and all tests were two-tailed. All statistical analyses were performed using SPSS 22.0 software.

Results

Patient demographics and clinical characteristics

In this study, *P. jirovecii* was identified in the BALF/blood of 34 patients by mNGS, who were diagnosed as PJP. Of these confirmed cases, 22 were male (64.7%) and 12 were female (35.3%). The mean age was 51.79 years (from 20 years to 84 years), 19 were kidney recipients, 1 liver recipient, 5 cases of connective tissue disease, 4 cases of blood system diseases, and 5 cases of others (including tumors and skin diseases). The detection results of sample types, the number reads of *P. jirovecii*, 1-3-β-D-glucan, and Methenamine silver staining are shown in Table 1. The patient had no significant adverse events following imaging studies, mNGS, and other diagnostic procedures.

mNGS information and related influencing factors

Among 34 patients who were diagnosed with PJP by mNGS, the number of reads mapped to *P. jirovecii* genome ranged from10 to 239032 and the mean number of reads for *P. jirovecii* was 2534. BALF/blood samples for mNGS were collected from 1 to 159 days after suspected patient infection, with a median collection time of 16.5 days, and it was found that there was no statistical difference. Based on the number of reads of *P. jirovecii*, we divided 34 patients into 2 groups, one group with reads ≤ 100(7 patients, 20.59%)and the other with reads>100(27 patients, 79.41%). Comparing the two groups, the duration from onset to sampling collection was generally shorter in patients with reads>100 than in those with reads ≤100 (8 days *vs.* 23 days, p=0.020). There was a statistically significant difference in the symptom of cough (7 persons *vs.* 14 persons, p=0.021),the symptom of sputum(5 persons *vs.* 7 persons, p=0.027), and clinical indicators such as C-reactive protein (15.8mg/L *vs.* 79.56mg/L, p=0.016), lactate dehydrogenase(696U/l, 494U/l, p=0.030) and procalcitonin (0.09ng/ml *vs.* 0.59ng/ml, p= 0.028) (Table 2).

Comparison of diagnostic value of mNGS, methenamine silver staining, and serum 1-3-β-D-glucan for PJP

Methenamine silver staining is the most commonly used clinical test for PJP, and serum 1-3-β-D-glucan is a widely used serological marker for PJP, if the value of 1-3-β-D-glucan is elevated, the patient may be infected with *P. jirovecii*. In the present study, we compared the diagnostic value of the mNGS, Methenamine silver staining and serum 1-3-β-D-glucan in 34 PJP patients. *P. jirovecii* was detected from patients by clinical microbiological testing methods, of which 34 patients were detected by Methenamine silver staining, only 1 was positive (2.9%) and 34 patients were detected by 1-3-β-D-glucan, and the value greater than 60 pg/mL was 67.65% (23/34). However, we detected *P. jirovecii* in 34 patients (100%) by mNGS. Thus the study shows that mNGS has a high detection rate for *P. jirovecii* infections, which also confirmed that the clinical mNGS performance is helpful for infection diagnosis.

Discussion

Currently, the incidence of PJP in non-HIV immunocompromised populations is increasing due to the prevalence of immunosuppressive diseases such as hematological malignancies, solid tumors, systemic corticosteroid therapy, immunosuppressive therapy, and organ transplantation (Gaborit et al., 2019). It has also been shown that in non-HIV-infected people, PJP usually develops into respiratory failure within a short period and results in a 30–60% mortality rate, which is significantly higher than in HIV-infected people (Cillóniz et al., 2019). Therefore, the early diagnosis of *P. jirovecii* infections in non-HIV patients is particularly important. In the past, the diagnosis of *P. jirovecii* was mainly based on staining, PCR, and detection of 1-3-β-D-glucan is a common cell wall constituent of most pathogenic fungi, including *P. jirovecii* (Lu et al., 2011a). But these methods have their limitations. Routine staining requires a large number of pathogens in the lungs and an experienced microbiologist to ensure the detection of *P. jirovecii* under the microscope;

10.3389/fcimb.2022.1026739

TABLE 1 Clinical microbiology results, sequencing information and demographic information of *P. jirovecii* positive patients.

Patient	Sex	Sample type	mNGS reads	Methenamine silver staining	1-3-β-D-glucan (pg/mL)	diagnosis
P1	M	BALF	162	–	683.2	kidney recipient
P2	M	BALF	217	–	10	others
P3	M	BALF	21745	–	10	blood system diseases
P4	F	BALF	20206	–	10	kidney recipient
P5	M	BALF	39	–	10	liver recipient
P6	M	BALF	12287	–	220.6	kidney recipient
P7	M	BALF	239032	–	10	kidney recipient
P8	M	Blood	13	–	118.6	kidney recipient
P9	F	BALFBlood	2169	–	158.2	kidney recipient
P10	F	Blood	131	–	156.8	kidney recipient
P11	F	Blood	378	–	202.9	kidney recipient
P12	M	BALF	1712	–	205.2	kidney recipient
P13	F	BALF	2754	–	877.5	kidney recipient
P14	M	BALF	3265	–	169.9	others
P15	F	BALF	16562	–	214.6	kidney recipient
P16	F	BALF	339	–	10	connective tissue disease
P17	F	BALFBlood	758	–	214.9	others
P18	M	BALF	60	–	234.4	kidney recipient
P19	M	BALF	3366	–	120.2	blood system diseases
P20	M	BALF	3070	–	263.5	kidney recipient
P21	M	BALF	15973	–	101.7	connective tissue disease
P22	M	BALF	8876	–	10	others
P23	M	BALF	2579	+	70.6	blood system diseases
P24	M	BALF	32447	–	10	kidney recipient
P25	M	BALF	38419	–	270.9	kidney recipient
P26	M	BALF	780	–	903.3	kidney recipient
P27	F	BALF	48	–	235.8	kidney recipient
P28	F	BALF	32	–	197.6	connective tissue disease
P29	M	BALF	2534	–	274.4	blood system diseases
P30	F	BALF	1808	–	467.1	connective tissue disease
P31	F	BALF	10	–	10	connective tissue disease
P32	M	BALF	2814	–	54.8	kidney recipient
P33	M	BALF	1850	–	287.1	kidney recipient
P34	M	BALF	22	–	10	others

M male, F female, ND not done, –negative, + positive, 1-3-β-D-glucan reference range:0-60.

therefore, it can be insensitive and biased (Procop et al., 2004). Studies have demonstrated that respiratory specimen PCR results are sufficient to confirm or rule out disease in high-risk patients with suspected *P. jirovecii* (Lu et al., 2011b), but it might fail value in single-shot detection of mixed infections, especially for those rare or novel strains (White et al., 2017). In addition, PCR and microarrays use specific primers or probes to target only one or a limited number of known pathogens, which is very inconvenient. However, for all DNA or RNA present in the sample, mNGS allows detection of the entire microbiome and host genome or transcriptome in patient samples (Camargo et al., 2019; Wang et al., 2019; Irinyi et al., 2020; Li et al., 2020). Besides, the non-specificity of serum 1-3-β-D-glucan limits its application in the diagnosis of PJP (Del Corpo et al., 2020). 1-3-β-D-glucan is a common cell wall constituent of most pathogenic fungi, including *P. jirovecii* (Lu et al., 2011a; Li et al., 2015). The 1-3-β-D-glucan assay has been approved for making a diagnosis of invasive fungal disease (De Pauw et al., 2008). However, the role of the serum-1-3-β-D-glucan assay in the diagnosis of PJP is controversial, especially among patients with or without HIV infections (Li et al., 2015). Studies have demonstrated that a negative result of the serum-1-3-β-D-glucan determination is sufficient to rule out PJP in HIV cases only, but in non-HIV patients, 1-3-β-D-glucan assays are insensitive and nonspecific for invasive fungal disease (Li et al., 2015; Del Corpo et al., 2020), thus, the results should therefore be carefully analyzed in parallel with clinical features, radiological findings, and other diagnostic evidence (Lu et al.,

TABLE 2 Comparison of different *P. jirovecii* reads abundance groups.

Item	mNGS reads ≤ 100 (N = 7)	mNGS reads >100 (N = 27)	P
Age (year)	55 (35,84)	49 (20,82)	0.565
Sex (female/male)	3/4	9/18	0.653
Fever (n/%)	4/57.14	19/70.37	0.511
Cough	7/100	14/51.85	0.021
Sputum	5/71.43	7/25.93	0.027
Dyspnea	2/28.57	13/48.15	0.360
Hemoptysis	0/0	1/3.7	0.611
Laboratory tests			
White blood cells ($\times10^9$/L)	7.75 (2.75,14.76)	9.48 (1.53,16.76)	0.949
Hemoglobin (g/L)	114 (63,141)	94 (58,142)	0.130
Platelet ($\times10^9$/L)	130 (111,351)	178 (23,487)	0.949
C-reactive protein (mg/L)	15.8 (3.09,74.8)	79.56 (4.28,227.3)	0.016
LDH (U/l)	696 (525,1110)	494 (0,1443)	0.030
PCT (ng/ml)	0.09 (0.05,5.54)	0.59 (0.05,28.74)	0.028
Duration from onset to BALF collection (days)	23 (11,37)	8 (1,159)	0.02

2011a; Li et al., 2015; Del Corpo et al., 2020). In conclusion, these methods will lead to the delay of clinical treatment and affect the prognosis of patients. Therefore, it is necessary to find a method with high diagnostic accuracy, short detection time, and accurate identification of infectious pathogens (Jiang et al., 2021). mNGS has an efficient workflow, relatively low-cost consumption, and short turnaround time. Hence mNGS may be widely accepted in clinical practice (Han et al., 2019). Moreover, mNGS also has the characteristics of high throughput and high sensitivity and can measure millions or even hundreds of millions of nucleic acid sequences at the same time, which plays a very important role in the accurate diagnosis of infectious diseases (Yu et al., 2011; Wilson et al., 2014; Ni et al., 2015; Tong et al., 2015).The diagnostic performance of mNGS in the respiratory tract (Langelier et al., 2018; Xie et al., 2019), bloodstream (Dubourg and Raoult, 2016), central nervous system (Ramachandran and Wilson, 2020), pleural cavity (Chen et al., 2021), and prosthetic joint infections (Thoendel et al., 2018) has been appreciated. Research data also supports its advantages in detecting opportunistic pathogens and co-infections, especially uncultivable pathogens (Parize et al., 2017; Pan et al., 2019), such as *Legionella pneumophila, Aspergillus* spp. (Yue et al., 2021), *Nocardia* spp. (Ding et al., 2021), *Mucor* spp. (Liu et al., 2021) etc.

In this study, clinical microbiology tests were compared with mNGS, and our data shows that the detection rate of mNGS for *P. jirovecii* in non-HIV infected PJP patients is significantly higher than that of serum 1-3-β-D-glucan and Methenamine silver staining, which can well auxiliary clinical diagnosis. Previous research shows that if the microorganism identified only by mNGS is accompanied by discrete evidence in clinical practice, the pathogen is considered a pathogen with a certain degree of clinical suspicion (Wu et al., 2020; Jiang et al., 2021). In our study, 34 patients with PJP were diagnosed through clinical comprehensive diagnosis and mNGS results, suggesting that mNGS plays an important role in assisting diagnosis of pathogens with a certain degree of clinical suspicion, which is consistent with the views in the above findings. We also analyzed the time interval from onset to the collection of samples and found that the interval time of the group with reads>100 was significantly shorter than that of the group with reads ≤ 100, which indicated that the number of reads detected by mNGS for *P. jirovecii* was closely related to the time of sample collection. This result is also consistent with previous studies, indicating that early treatment, effective use of antibiotics, and improved disease status will reduce the number of reads detected by mNGS for pathogens (Ai et al., 2018; Zhang Xx et al., 2019).

In addition, we compared some factors that may affect the reads abundance of *P. jirovecii* detected by mNGS. The results of the univariate analysis showed that age, gender, white blood cells, hemoglobin, and platelets did not affect the reads abundance of *P. jirovecii*, while C-reactive protein, LDH, and procalcitonin were significantly different between the group with reads>100 and the group with reads ≤ 100.These results suggest that concurrent detection of inflammatory status during mNGS may determine the pathogen abundance, which is also consistent with previous studies (Zhang Xx et al., 2019).

This study also has certain limitations. Firstly, due to the small sample size, the positive rate of clinical routine method evaluation is low, so the sensitivity of mNGS may be slightly overestimated which belongs to partial validation bias. Secondly, because BALF is adopted at different positions of lung segment, the partial deviation will occur, which will affect the reads abundance of *P. jirovecii* accompanied with the sensitivity evaluation. Thirdly, due to limited conditions, the hospital did not routinely perform PCR for *P. jirovecii* identification, so the diagnostic performance of

mNGS and PCR was not compared. Finally, mNGShas disadvantages, for example, it is difficult for mNGS to distinguish *P. jirovecii* colonization from infection because there is no widely accepted mNGS quantification cutoff or threshold. In addition, the sequencing depth of mNGS is still limited, and the pathogen database needs further improvement (Gargis et al., 2016). Therefore, a definitive diagnosis of PJP must be based on a comprehensive summary of clinical features, laboratory abnormalities, imaging findings, and microbiological evidence, rather than mNGS alone (Jiang et al., 2021). However, the rapid development of next-generation sequencing technology will show higher sensitivity and specificity in diagnosing infections in the future (Thorburn et al., 2015), and we are confident that the mNGS will effectively give a valuable performance in clinical infection diagnosis.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://bigd.big.ac.cn/gsa/browse/CRA008494 and the assigned accession of the submission is: CRA008494.

Ethics statement

This study was reviewed and approved by Medical ethics committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to the study conception and design. MZ, RY, LP, and MH wrote the manuscript draft. RY, XW, and ZG conducted most of the experiments. MZ and MH performed data analysis. LP and MH conceived the idea and directed the experiments. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by Research and Development Projects of Science and Technology Department of Sichuan Province (Grant No.2019YFS0319), Sansure Biotech Transfusion Medicine Development Fund of Chinese Society of Blood Transfusion (CSBT-SX-2021-01) and Project of the Special Fund for Young and Middle aged Medical Research of the China International Medical Exchange Foundation(Z-2018-35-19202).

Acknowledgments

We acknowledge Dr. Fan Zhenxin (College of Life Sciences, Sichuan University) for bioinformatics helping, and Mr. Wang Baoshan (Mountain in Sight (Sichuan) Medical Technology Co. Ltd) for coordinating sample collection and shipping.

Conflict of interest

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

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Supplementary material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 10 November 2022
DOI 10.3389/fcimb.2022.1042945

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 13 September 2022
ACCEPTED 24 October 2022
PUBLISHED 10 November 2022

CITATION
He P, Wang J, Ke R, Zhang W, Ning P, Zhang D, Yang X, Shi H, Fang P, Ming Z, Li W, Zhang J, Dong X, Liu Y, Zhou J, Xia H and Yang S (2022) Comparison of metagenomic next-generation sequencing using cell-free DNA and whole-cell DNA for the diagnoses of pulmonary infections. *Front. Cell. Infect. Microbiol.* 12:1042945.
doi: 10.3389/fcimb.2022.1042945

Comparison of metagenomic next-generation sequencing using cell-free DNA and whole-cell DNA for the diagnoses of pulmonary infections

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Although the fast-growing metagenomic next-generation sequencing (mNGS) has been used in diagnosing infectious diseases, low detection rate of mNGS in detecting pathogens with low loads limits its extensive application. In this study, 130 patients with suspected pulmonary infections were enrolled, from whom bronchoalveolar lavage fluid (BALF) samples were collected. The conventional tests and mNGS of cell-free DNA (cfDNA) and whole-cell DNA (wcDNA) using BALF were simultaneously performed. mNGS of cfDNA showed higher detection rate (91.5%) and total coincidence rate (73.8%) than mNGS of wcDNA (83.1% and 63.9%) and conventional methods (26.9% and 30.8%). A total of 70 microbes were detected by mNGS of cfDNA, and most of them (60) were also identified by mNGS of wcDNA. The 31.8% (21/66) of fungi, 38.6% (27/70) of viruses, and 26.7% (8/30) of intracellular microbes can be only detected by mNGS of cfDNA, much higher than those [19.7% (13/66), 14.3% (10/70), and 6.7% (2/30)] only detected by mNGS of wcDNA. After in-depth analysis on these microbes with low loads set by reads per million (RPM), we found that more RPM and fungi/viruses/intracellular microbes were detected by mNGS of cfDNA than by mNGS of wcDNA. Besides, the abilities of mNGS using both cfDNA and wcDNA to detect microbes with high loads were similar. We highlighted the advantage of mNGS using cfDNA in detecting fungi, viruses, and intracellular microbes with low loads, and suggested that mNGS of cfDNA could be considered as the first choice for diagnosing pulmonary infections.

KEYWORDS

MNGs, cell-free DNA, whole-cell DNA, pulmonary infection, BALF

Introduction

Pulmonary infections are highly prevalent diseases with considerable morbidity and mortality in individuals of all ages (Griffin et al., 2013; Collaborators GBDCoD, 2017). As the third leading causes of years of life lost, about 2.4 million patients per year died of pulmonary infections globally (Collaborators GBDCoD, 2017). Various pathogens can cause such infections (Kradin and Digumarthy, 2017; Lin et al., 2021), with the presenting symptoms of fever, cough, sputum production, dyspnoea, pleuritic chest pain, and so on (Ruiz et al., 2000). However, due to the similar clinical manifestations among patients infected by different kinds of pathogens, the accurate and timely etiological diagnosis are always difficult for clinicians (Cunha, 2006; Sheu et al., 2010). Conventional tests for diagnoses of pulmonary infections, including time-consuming culture methods with low positive rate, polymerase chain reaction (PCR) based on prior hypothesis of the target, and serology tests with interpretational difficulties, are not satisfactory (Carroll and Adams, 2016; Buchan et al., 2021). Delay and misdiagnosis of pulmonary infections can lead to disease progression, resulting in worse prognoses and even death (Garnacho-Montero et al., 2018). More rapid and accurate methods for clinical diagnoses of pulmonary infections are needed.

Unbiased metagenomic next-generation sequencing (mNGS) has been increasingly applied in diagnosing multiple infectious diseases, such as meningitis and sepsis (Chiu and Miller, 2019; Gu et al., 2021), exhibiting significant advantages over conventional methods (Chen et al., 2021; Chen et al., 2021). mNGS of both cell-free DNA (cfDNA) and whole-cell DNA (wcDNA) are being used (Han et al., 2020). Compared to wcDNA extraction, cfDNA is extracted from extremely low-cellularity supernatant of samples (Szilagyi et al., 2020). Besides, cfDNA extraction is considered to decrease the load of pathogen DNA in the sample, especially for intracellular pathogens, but it can avoid DNA degradation caused by processes that wcDNA extraction requires. However, few investigations have been performed to evaluate diagnostic values of mNGS using cfDNA and wcDNA.

In this study, a total of 130 patients with suspected pulmonary infections were enrolled. The remaining bronchoalveolar lavage fluid (BALF) samples from those patients were used for mNGS of cfDNA and wcDNA. Our aim was to evaluate performance of mNGS using BALF in the clinical diagnosis of pulmonary infections against conventional methods, and effectiveness of mNGS using cfDNA and wcDNA was also compared.

Methods

Patient recruitment and study design

Patients with suspected pulmonary infections admitted to Department of Pulmonary and Critical Care Medicine of The Second Affiliated Hospital of Xi'an Jiaotong University from September 2019 to September 2021 were enrolled. The diagnosis of pulmonary infection was based on 1) new-onset radiological findings on chest X-ray or computed tomography (CT) and 2) at least one of the following typical clinical characteristics: a) new-onset cough, sputum production, dyspnoea, chest pain, or exacerbation of existing respiratory symptoms; b) fever; c) clinical signs of lung consolidation or moist rales; d) peripheral leukocytosis (>10×10^9/L) or leucopenia (<4×10^9/L).

Conventional diagnostic tests and mNGS were performed simultaneously. Conventional diagnostic tests used in this study included culture, antibody measurement, PCR, Xpert, and pulmonary histopathology. Physical information and clinical details were investigated. Remaining BALF sample from each enrolled patient was collected into a 5 mL sterile tube transported to Hugobiotech (Hugobiotech, Beijing, China) immediately for mNGS of cfDNA and wcDNA.

Sampling and mNGS sequencing

According to manual of QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany), cfDNA and wcDNA were extracted. For cfDNA extraction, BALF supernatant obtained by centrifugation was used for subsequent protocols, while BALF sample was directly used for wcDNA extraction (bead-beating method) without centrifugation. Before library construction (QIAseq Ultralow Input Library Kit, QIAGEN, Hilden, Germany), we tested concentration and quality of cfDNA and wcDNA using Qubit 4.0 (Thermo Fisher Scientific, MA, USA). Qualified libraries were sequenced on Nextseq 550 platform (Illumina, San Diego, USA). Negative controls using sterile deionized water and positive controls using synthesize fragments with known quantities were established for each batch of experiments using the same wet lab procedures and bioinformatics analysis as the clinical samples.

Bioinformatics pipeline

Clean reads were obtained by removing adapter and low-quality and short reads (<35bp) from raw data generated by sequencing. Human sequences were excluded by mapping to the human reference genome (hg38) using bowtie2. The remaining clean reads were then blasted against a microbial Pan-genome database which was constructed based on the published microbial genome databases, including database of National Center for Biotechnology Information (ftp://ftp.ncbi.nlm.nih.gov/genomes/).

Statistical analysis

Medians, interquartile ranges (IQRs), and 95% confidence interval (CI) were calculated using IBM SPSS 25.0. Sensitivity,

specificity, positive predicative value (PPV), negative predicative value (NPV), and total coincidence rate (TCR) of mNGS were calculated against clinical diagnoses. Bar charts and heatmap were generated using R 4.1.1. Comparison with P value of < 0.05 was considered statistically significant. The number of microbes and base-2 logarithm of reads per million (RPM) detected by mNGS of cfDNA divided by that detected by mNGS of wcDNA for the same microbe were used to draw scatter plot, and the dots of >0 represented that the numbers of RPM detected by mNGS of cfDNA were higher than those detected by mNGS of wcDNA for the microbes. We set different thresholds for RPM (RPM of 25, 50, 100, 200, and 500) to divide the microbes into groups of high and low loads. The microbe with the number of RPM detected by mNGS of cfDNA or wcDNA higher than the threshold was divided into the high load group, while the others were divided into low load group.

Results

Patient characteristics

A total of 130 patients were enrolled in our study, including 81 males and 49 females. The age of these patients ranged from 20 to 67 years old, with a median age of 54 years old (Table 1). Most of patients (86.1%, 112/130) had underlying diseases, such as cardiovascular diseases (21), cerebrovascular diseases (13), hepatopathy (24), tumors (29), diabetes (18), autoimmunity disease (11), and anemia (13). According to mNGS results, conventional tests, and therapeutic effects, 121 and 9 patients were finally diagnosed as infectious and non-infectious diseases, respectively. The most common microbes detected were bacteria (85/121), followed by fungi (27/121) and viruses (15/121). Besides, co-infections were found in 21 patients (Table 2).

TABLE 1 Clinical characteristic of enrolled patients.

Clinical characteristics		Count (percent) / Median(IQR)[min,max] (95% CI)
Gender	Male	81 (62.3%)
	Female	49 (37.7%)
Age		54 (20.75)[20,67](50.8~56.2)
Cough		112 (86.1%)
Expectoration		98 (75.4%)
Dyspnea		74 (56.9%)
Hydrothorax		17 (10%)
Intensive care unit		6 (4.6%)
Underlying diseases		112 (86.1%)
Autoimmunity disease		11 (8.5%)
Cardiovascular disease		21 (16.2%)
Cerebrovascular disease		13 (10%)
Diabetes		18 (13.8%)
Chronic obstructive pulmonary disease		8 (6.2%)
Pulmonary sarcoidosis		3 (2.3%)
Nephrosis		9 (6.9%)
Hepatopathy		24 (18.5%)
Asthma		7 (5.4%)
Tumors		29 (22.3%)
Anemia		13 (10%)

IQR, interquartile ranges; CI, confidence interval.

Potential pathogen profiles

mNGS of cfDNA identified bacteria (n=142), fungi (n=60), viruses (n=53), mycoplasmas (n=7), chlamydiae (n=2), and rickettsiae (n=2), most of which were also detected by mNGS of wcDNA (134 bacteria, 45 fungi, 43 viruses, 5 mycoplasmas, 2 chlamydiae, and 1 rickettsiae) (Figures 1A, B). Besides, the dominant microbes identified by mNGS of cfDNA and wcDNA were almost accordant. The main bacteria were *Pseudomonas aeruginosa*, *Haemophilus parainfluenzae*, *Haemophilus influenza*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, and *Escherichia coli*, and fungi were *Candida albicans*, *Pneumocystis jirovecii*, and *Aspergillus fumigatus*. A total of 8 viruses were detected, with the detection of Human betaherpesvirus 5 and Human gammaherpesvirus 4 at the highest frequencies (Figure 1C).

However, some bacteria (2: *Moraxella catarrhalis* and *Nocardia farcinica*), fungi (5: *Aspergillus nidulans*, *Aspergillus versicolor*, *Penicillium citrinum*, *Rhizopus delemar*, and *Rhizopus oryzae*), and viruses (3: Human polyomavirus 10, Human polyomavirus 2, and Human polyomavirus 6), and *Mycobacterium avium* were only detected by mNGS of cfDNA. Although there were no significant difference in overall detection of microorganisms, more microbes identified by mNGS of cfDNA can provide more effective reference for clinicians.

mNGS performance

From case perspective, the performance of mNGS using cfDNA was better than that of mNGS using wcDNA. Conventional methods only detected microorganisms from 26.9% of BALF samples (35/130). Conversely, detection rate of mNGS using cfDNA reached 91.5% (119/130), higher than that

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TABLE 2 The types of infections of enrolled patients based on clinical diagnosis.

Infections	Case number
Bacteria	66
Fungi	14
Virus	3
Bacteria + Fungi	8
Bacteria + Virus	6
Fungi + Virus	1
Bacteria + Fungi + Virus	4
Mycoplasma	3
Chlamydia	2
Bacteria + Mycoplasma	1
Mycoplasma + Chlamydia + Virus	1
Indefinite clinical diagnosis	12
Non-infected	9

of mNGS using wcDNA (108/130, 83.1%). Besides, sensitivity (76.9%), specificity (44.4%), PPV (94.9%), and NPV (12.5%) of mNGS using cfDNA were all higher than those (66.1%, 33.3%, 93%, and 6.8%) of mNGS using wcDNA (Figure 2B). Most importantly, TCR of mNGS using cfDNA against final clinical diagnoses was 73.8%, higher than those of mNGS using wcDNA (63.9%) and conventional methods (30.8%) (Figure 2A).

Given mNGS advantages and microbial complexity, we further evaluated the efficiencies of mNGS in detecting bacterial, fungal, and viral infections. Among the 85 patients with bacterial infection, mNGS of cfDNA (89.4%) and wcDNA (83.5%) respectively detected microbes from 76 and 71 patients (Figure 2C). However, mNGS of cfDNA exhibited better performance in detecting both fungi (88.9%, 24/27) and viruses (93.3%,14/15) than mNGS of wcDNA (66.7% and 53.3%, respectively) (Figure 2C). These results further indicate that mNGS of cfDNA is much more suitable for diagnosing

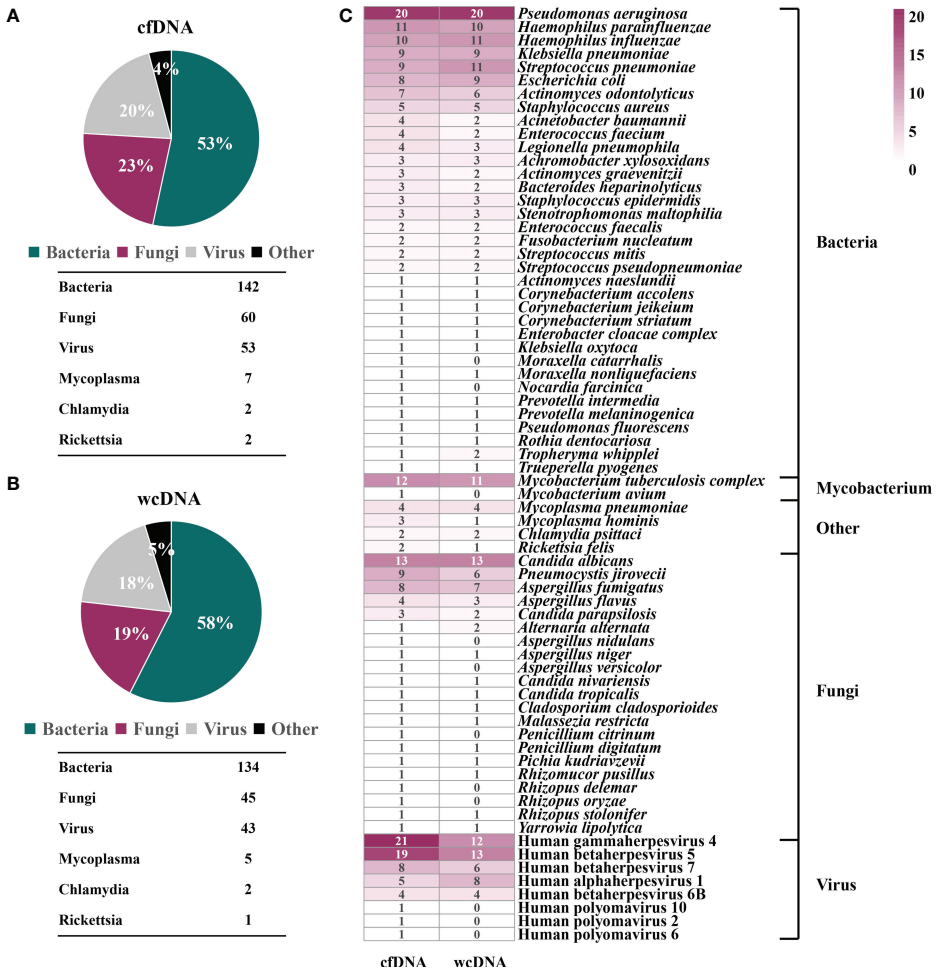

FIGURE 1
Potential pathogen profiles detected by mNGS of cfDNA and wcDNA. **(A)**, Microbial composition revealed by mNGS of cfDNA. **(B)**, Microbial composition revealed by mNGS of wcDNA. **(C)**, Comparison of mNGS using cfDNA and wcDNA in detecting microorganisms. The number in the box is the total number of patients from which some microorganism was detected.

pulmonary infections than mNGS of wcDNA, driving us to dig out in-depth reasons from perspectives of total microorganisms and RPM detected.

Differences in numbers of RPM detected

To evaluate the detection efficiencies of mNGS, comparison of difference in detected RPM between mNGS of cfDNA and wcDNA was further performed by infection types. There were 206 microbes detected by mNGS of both cfDNA and wcDNA. For most of microbes (54.3%), mNGS of cfDNA detected more RPM than mNGS of wcDNA (Figure 3A). Similar trends were also found in detecting bacteria (54.8%), fungi (56.3%), and viruses (51.5%) (Figures 3B-D). These results show that mNGS of cfDNA can capture more reads from most of microbes than mNGS of wcDNA.

Differences in clinical diagnostic value between the two methods are caused by the heterogeneity of microbes detected, rather than the uniformity. Among the 308 microbes, 69 (22.4%) and 33 (10.7%) microbes were only detected by mNGS of cfDNA and wcDNA, respectively (Table 3). The 31.8% (21/66) of fungi, 38.6% (27/70) of viruses, and 26.7% (8/30) of intracellular microbes (viruses were not included) were only detected by mNGS of cfDNA, much higher than those (19.7% (13/66), 14.3% (10/70), and 6.7% (2/30), respectively) by mNGS of wcDNA (Table 3). Besides, the number of RPM only detected by mNGS of cfDNA ranged from 3 to 12010, lower than that (from 2 to 30446) by mNGS of wcDNA. These results indicate that the better performance of mNGS using cfDNA might be caused by the successful detection of microbes with low loads, especially for fungi, viruses, and intracellular microbes.

Identifying microbes with low loads

Based on the assumption that sequencing process did not influence the detection of mNGS using both cfDNA and wcDNA, we proposed that the level of microbial loads can be directly reflected by the numbers of RPM. Accordingly, we set different thresholds for RPM to evaluate the ability of mNGS using cfDNA and wcDNA to detect microbes. For bacteria with high loads at different thresholds (RPM of 100, 200, and 500), mNGS of cfDNA detected more RPM than mNGS of wcDNA in 58.0%-63.7% of bacteria, while the two methods had the similar abilities to detect bacteria with low loads (Figure 4 and Table S1).

Better performance of mNGS using cfDNA was also observed in detecting fungi and viruses with low loads. For fungi with low loads at different thresholds (RPM of 25, 50, 100, and 200), the numbers of RPM from about 60% of fungi detected by mNGS of cfDNA were higher than those detected by mNGS of wcDNA. Most importantly, the number of fungi only detected by mNGS of cfDNA was about one time higher than that by mNGS of wcDNA. However, no significant difference in the number of RPM between mNGS of cfDNA and wcDNA was found in detecting fungi with high loads (Figure 4 and Table S1). A similar trend was found in detecting viruses. These results unravel that the better performance of mNGS using cfDNA is definitely caused by the successful detection of fungi and viruses with low loads.

Identifying intracellular microbes

Given the long-held conflict in location of proliferation and infection between intracellular and extracellular pathogens, we summarized the performance of mNGS in detecting intracellular microbes (viruses were not included). From 27 patients, *Mycobacterium tuberculosis* complex, *Mycoplasma hominis*, *Legionella pneumophila*, *Chlamydia psittaci*, and *Rickettsia felis* were detected with total number of 30. The detection rate of mNGS using cfDNA was 92.6% (25/27), slightly higher than that of mNGS using wcDNA (81.5%, 22/27). About 26.7% and 6.7% of intracellular microbes were only detected by mNGS of cfDNA and wcDNA, respectively (Table 3).

For intracellular microbes with both high and low loads at different thresholds (RPM of 25, 50, 100, 200, and 500), the numbers of RPM from 50%-77.8% of mircobes detected by mNGS of cfDNA were higher than those detected by mNGS of wcDNA (Figure 4 and Table S1). Besides, the number of microbes with low loads only detected by mNGS of cfDNA was more than two times higher than that only detected by mNGS of wcDNA. Interestingly, all of intracellular microbes with high loads at different thresholds detected by mNGS of wcDNA were detected by mNGS of cfDNA, but some intracellular microbes (species number of 1-3) detected by mNGS of cfDNA cannot be detected by mNGS of wcDNA at all. The above results provided sufficient evidences for the better performance of mNGS using cfDNA in detecting intracellular microbes, especially for those with low loads.

Discussion

This is the first report on evaluating performance of mNGS using cfDNA and wcDNA of BALF samples in diagnosing pulmonary infections. The highest detection rate (91.5%) and TCR (73.8%) were found by mNGS of cfDNA, followed by mNGS of wcDNA (83.1% and 63.9%) and conventional methods (26.9% and 30.8%). We provided sufficient evidences for that the better performance of mNGS using cfDNA than that of mNGS using wcDNA is definitely caused by the successful detection of microbes with low loads, especially for fungi, viruses, and intracellular microbes.

To evaluate the performance of mNGS in diagnosing pulmonary infections, mNGS tests were performed using

A

	Detection Rate		Total Coincidence Rate		Positive Coincidence Rate		Negative Coincidence Rate	
Conventional Methods	35/130	(26.9%)	40/130	(30.8%)	31/35	(88.6%)	9/95	(9.5%)
mNGS of cfDNA	119/130	(91.5%)	96/130	（73.8%）	92/119	(77.3%)	4/11	(36.4%)
mNGS of wcDNA	108/130	(83.1%)	83/130	（63.9%）	80/108	(74.1%)	3/22	(13.6%)

B

	Clinical Diagnosis	
Conventional Methods	+	-
+	31	0
-	93	9

Sensitivity	25.6%
Specificity	100%
PPV	100%
NPV	9.1%

	Clinical Diagnosis	
mNGS of cfDNA	+	-
+	92	5
-	29	4

Sensitivity	76.0%
Specificity	44.4%
PPV	94.9%
NPV	12.5%

	Clinical Diagnosis	
mNGS of wcDNA	+	-
+	80	6
-	41	3

Sensitivity	66.1%
Specificity	33.3%
PPV	93%
NPV	6.8%

C

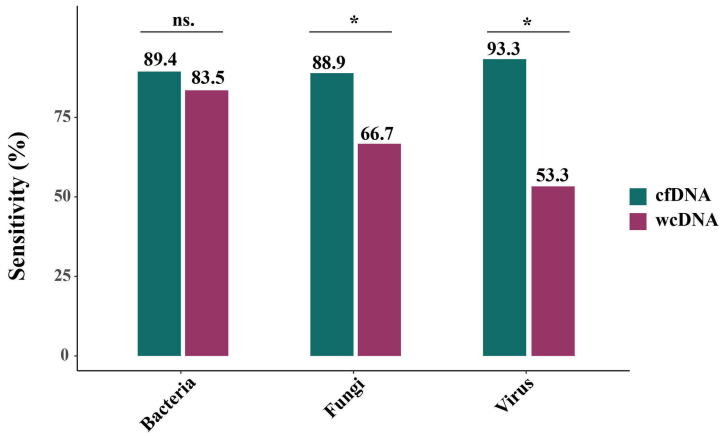

FIGURE 2
Performance of mNGS using cfDNA/wcDNA and conventional methods. **(A, B)** show the coincidence rates, sensitivity, specificity, PPV, and NPV of conventional methods, mNGS of cfDNA, and mNGS of wcDNA. PPV and NPV represent positive predictive value and negative predictive value, respectively. **(C)**, Comparison of mNGS using cfDNA and wcDNA at bacterial, fungal, and viral levels. *P < 0.05; ns: no significance.

cfDNA and wcDNA without host depletion. Host depletion methods, such as differential lysis method, can filter human DNA (Ji et al., 2020), increasing pathogen DNA ratio (Thoendel et al., 2018; Ji et al., 2020; Gu et al., 2021) at the expense of some viruses, parasites, and bacteria (Ji et al., 2020). Besides, host depletion methods lose the cfDNA in supernatant, bring in contamination of engineered strains from reagents (Gu et al., 2021), and decrease the detection rates of pathogens (Han et al.,

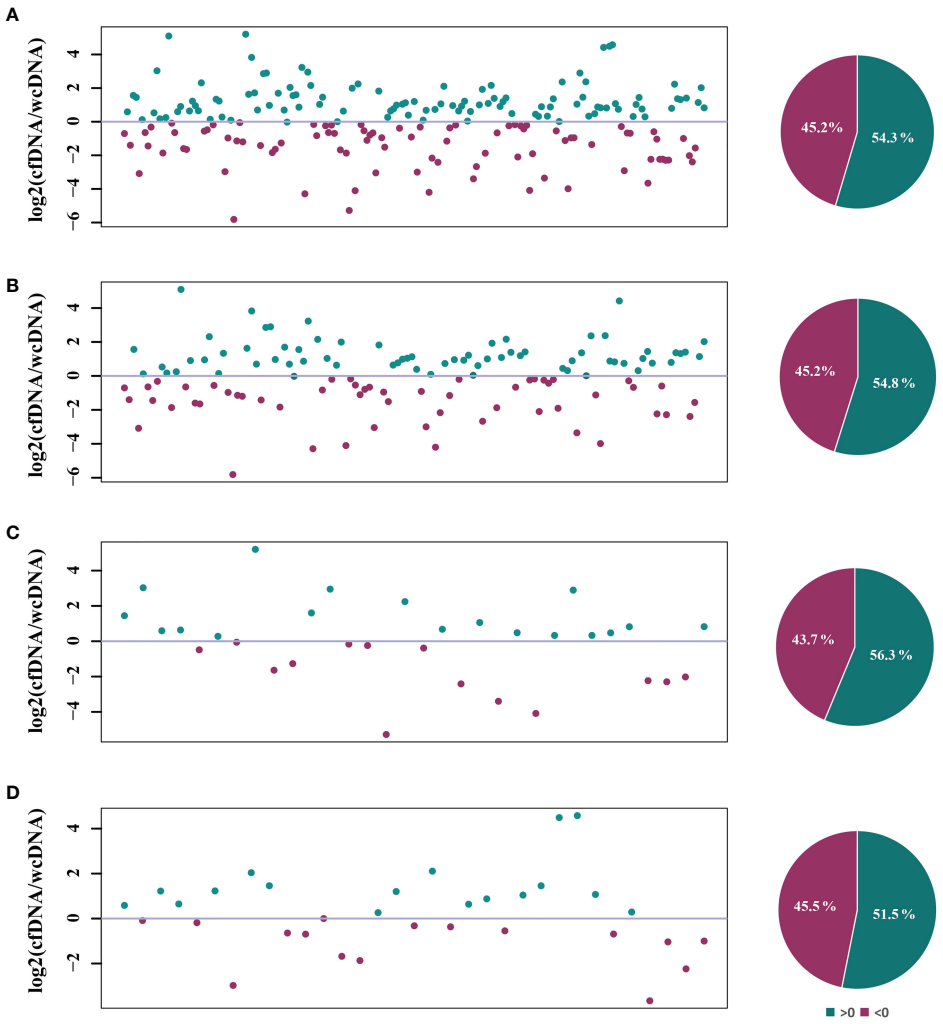

FIGURE 3
Comparison of the RPM detected by mNGS of both cfDNA and wcDNA. **(A)**, All microbes detected by mNGS. **(B)**, Bacteria detected by mNGS. **(C)**, Fungi detected by mNGS. **(D)**, Viruses detected by mNGS. The number of microbes and base-2 logarithm of reads per million (RPM) detected by mNGS of cfDNA divided by that detected by mNGS of wcDNA for the same microbes were used to draw scatter plot, and the dots of >0 represented that the numbers of RPM detected by mNGS of cfDNA was higher than that detected by mNGS of wcDNA for the microbes. The microbes only detected by mNGS of cfDNA or wcDNA were summarized in Table 3.

TABLE 3 Statistics of microbes only detected by mNGS of cfDNA or wcDNA.

	Only detected	All Microbes	Bacteria	Fungi	Viruses	Intracellular Microbes
Count	**by mNGS of cfDNA**	69/308	18/152	21/66	27/70	Aug-30
(Percentage)		-22.40%	-11.80%	-31.80%	-38.60%	-26.70%
RPM		(3~12010)	(6~12010)	(3~92)	(4~67)	(4~1875)
Count	**by mNGS of wcDNA**	33/308	10/152	13/66	Oct-70	Feb-30
(Percentage)		-10.70%	-6.60%	-19.70%	-14.30%	-6.70%
RPM		(2~30446)	(6~30446)	(7~851)	(2~843)	(6, 17)

2021). The long-held conflict in location of proliferation and infection between intracellular and extracellular pathogens (Casadevall and Fang, 2020) challenged whether mNGS of cfDNA can accurately detect causative pathogens. Accordingly, mNGS tests of wcDNA without host depletion used in this study can be considered as a suitable contrast to the mNGS tests of cfDNA, and the use of the both tests ensures reliability and accuracy of our data.

mNGS of cfDNA exhibited better performance in diagnosing pulmonary infections than mNGS of wcDNA and conventional methods. Low detection efficiencies and accuracies of conventional methods, such as culture (Rong et al., 2020) and antibody test (Shibata et al., 2020), hindered accurate and comprehensive detection of pathogens. Conversely, mNGS of cfDNA has been proven to be a promising tool for detecting pathogens in body fluids with high sensitivities (75-91%) and specificities (81-100%) (11). In addition, the sensitivity of mNGS is determined by pathogen DNA ratio in sample. However, wcDNA extraction from BALF sample by cell fragmentation without host depletion must increase the release of human DNA (11). CfDNA was directly extracted from low-cellularity supernatant of BALF samples (14), resulting in that pathogen DNA ratio of cfDNA might be higher than that of wcDNA for the same BALF sample, and the sensitivity of mNGS using BALF cfDNA reached up to about 90% (14). The above may be the reasons why the performance of mNGS using cfDNA is better than that of mNGS using wcDNA and conventional methods.

Obvious advantage of mNGS using cfDNA over mNGS using wcDNA is reflected in detecting pathogens with low loads, rather than high loads. We propose that the better performance of mNGS using cfDNA is attributed to its advantage in DNA extraction and bioinformatics analysis for trace pathogens. Although sample pretreatment for host depletion is not included in this study, wcDNA extraction involves cell wall lysis (Wilson et al., 2019), increasing the risk of DNA degradation (Thoendel et al., 2018; Gu et al., 2021) and decreasing DNA recovery rate of pathogens with low loads (Han et al., 2021), rather than high loads. Mild extraction of cfDNA without cell wall lysis should have few effect on the DNA recovery, which has been confirmed by successful detection of trace pathogens from cerebrospinal fluid using mNGS of cfDNA (Ji et al., 2020).

Furthermore, high human DNA ratio can reduce the denoising performance of bioinformatics algorithm in dry lab pipeline of mNGS and influence subsequent pathogen identification (Miller et al., 2019), especially for trace pathogens (Ji et al., 2020), which was confirmed by low detection rate (~50%) (Zhang et al., 2020) and coincidence rate (~50%) (Wilson et al., 2019) of mNGS using CSF wcDNA without host depletion and high sensitivity (>90%) of mNGS using cfDNA (Gu et al., 2021). Besides, accuracy of bioinformatics algorithm used in this study has been demonstrated by successful identification of 2 reads from desired pathogen using mNGS of wcDNA with host depletion (Wu et al., 2020). Equipped with the same bioinformatics algorithm, trace *M. tuberculosis* (RPM: 2.28) (Ji et al., 2020) was identified by mNGS of cfDNA, rather than mNGS of wcDNA. Lower DNA recovery rate and worse denoising performance of mNGS using wcDNA without host depletion comprehensively reduce its accuracy in detecting trace pathogens.

mNGS of cfDNA can be fully competent for detecting fungal and intracellular pathogens, challenging the opinion that process of cell wall lysis, such as bead-beating process, is necessary for DNA extraction (wcDNA) to ensure mNGS detection for those pathogens (Casadevall and Fang, 2020; Han et al., 2021). Adding bead-beating process was reported to significantly improve the detection of *Aspergillus fumigatus* by mNGS of wcDNA (Han et al., 2021). However, with the host immune attack (Arciola et al., 2018) and microbial autolysis (Wolf et al., 2017), DNA of intracellular and fungal pathogens can be released into body fluids in the form of cfDNA (Casadevall and Fang, 2020). Besides, we previously detected fungal (including *Aspergillus*) and intracellular pathogens consistent with clinical diagnosis from BALF samples using mNGS of cfDNA (Chen et al., 2021). Accordingly, extensive application of mNGS using cfDNA in diagnosing pulmonary infections could be expected.

Limitations

Firstly, multi-center study should be performed to provide more effective data and avoid intrinsic bias. Secondly, mNGS of wcDNA with host depletion should be included to capture more difference caused by different sample processing methods. Thirdly, efficiency of mNGS using cfDNA in detecting mycobacteria and intracellular microbes can be further investigated on large scale. Furthermore, more detailed clinical information, such as therapeutic regimens before and after mNGS, can be collected to evaluate implications of mNGS for clinical reference.

Conclusions

mNGS exhibited higher sensitivities and coincidence rates against clinical diagnosis than conventional methods in detecting microbes from patients with pulmonary infections. We emphasized the advantage of mNGS using BALF cfDNA with sufficient evidences in detecting microbes with low loads,

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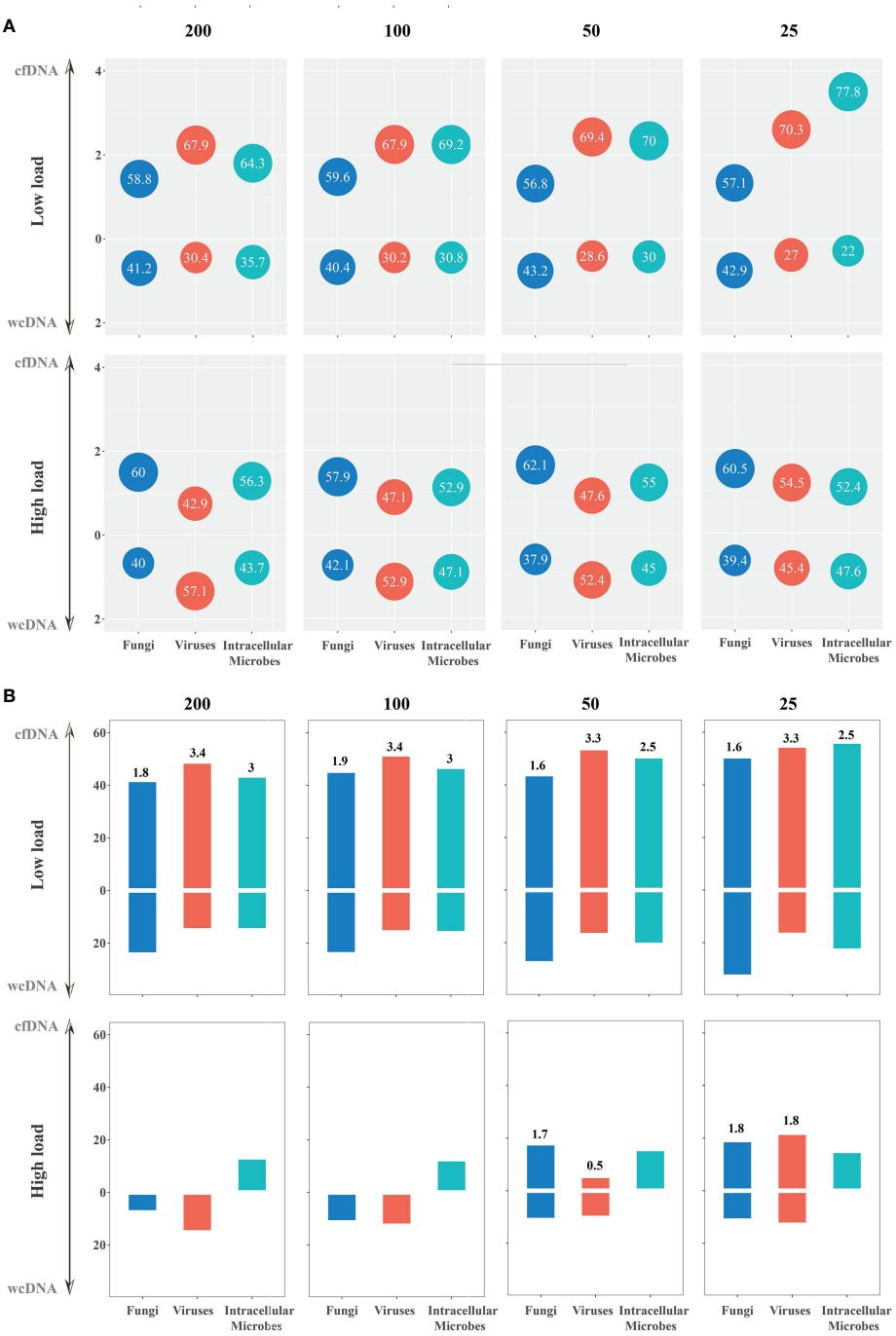

FIGURE 4
The detection of mNGS using cfDNA and wcDNA at different thresholds (RPM of 200, 100, 50, and 25). **(A)** Detection summary of fungi, viruses, and intracellular microbes by mNGS of both cfDNA and wcDNA at high and low loads. The number in the bubble above the horizontal axis is the ratio of the microbes with more RPM by mNGS of cfDNA than mNGS of wcDNA to the whole microbes at this threshold, while the number in the bubble below the horizontal axis is the ratio of the microbes with more RPM by mNGS of wcDNA than mNGS of cfDNA. Besides, vertical axis coordinate is the ratio of the numbers in the bubble. **(B)** Detection summary of fungi, viruses, and intracellular microbes only detected by mNGS of cfDNA or wcDNA at high and low loads. The column is the ratio of the microbes only detected by mNGS of cfDNA or wcDNA to the whole microbes at this threshold. The number above the column is the ratio of the microbes only detected by mNGS of cfDNA divided by that only detected by mNGS of wcDNA.

especially for fungi, viruses, and intracellular microbes, and its extensive application in diagnosing pulmonary infections could be excepted.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://ngdc.cncb.ac.cn/?lang=zh, PRJCA008662.

Ethics statement

The studies involving human participants were reviewed and approved by the Clinical Research Ethics Committee of the second affiliated hospital of Xi'an Jiaotong University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SY and HX designed the paper. PH and JW drafted the manuscript. RK, WZ, PN, DZ, XY, HS, PF, ZM, WL, JZ, XD and YL carried out the clinical care and management of the patients and performed the mNGS tests. JMZ analyzed the data. SY and HX revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by Shaanxi Qinchuangyuan "Scientists and Engineers" Team Construction Project (No. 2022KXJ-82).

Conflict of interest

JW, JMZ and HX were employed by Hugobiotech.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1042945/full#supplementary-material

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TYPE Original Research
PUBLISHED 13 January 2023
DOI 10.3389/fcimb.2022.1094518

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 10 November 2022
ACCEPTED 28 December 2022
PUBLISHED 13 January 2023

CITATION
Liu Y, Zhang R, Yao B, Yang J, Ge H, Zheng S, Guo Q and Xing J (2023) Metagenomics next-generation sequencing provides insights into the causative pathogens from critically ill patients with pneumonia and improves treatment strategies.
Front. Cell. Infect. Microbiol. 12:1094518.
doi: 10.3389/fcimb.2022.1094518

Metagenomics next-generation sequencing provides insights into the causative pathogens from critically ill patients with pneumonia and improves treatment strategies

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Background: The metagenomics next-generation sequencing (mNGS) is a promising technique for pathogens diagnosis. However, whether the application of mNGS in critically ill patients with pneumonia could cause anti-infection treatment adjustment and thereby affect the prognosis of these patients has not been explored.

Methods: We retrospectively collected the clinical data of patients diagnosed with pulmonary infection in the ICU of the Affiliated Hospital of Qingdao University from January 2018 to January 2021. These patients with pneumonia were divided into mNGS group and no-mNGS group by whether being performed NGS or not. The clinical data, including demographics, illness history, APACHE II score, length of mechanical ventilation, length of stay in the hospital, length of stay in ICU and outcome, were collected. In addition, the data of pathogens and anti-infection treatment before and after NGS were also collected. Propensity score matching was performed to evaluate the mortality and deterioration rate between NGS group and non-NGS group.

Results: A total of 641 patients diagnosed with pneumonia were screened, and 94 patients were excluded based on exclusion criteria. Finally, 547 patients were enrolled, including 160 patients being performed NGS. Among these 160 patients, 142 cases had NGS-positive results. In addition, new pathogens were detected in 132 specimens by NGS, which included 82 cases with virus, 18 cases with fungus, 17 cases with bacteria, 14 cases with mycoplasma, and 1 case with mycobacterium tuberculosis. Anti-infection treatments were adjusted in some patients who performed NGS, including 48 anti-bacterial treatments, 20 antifungal treatments and 20 antiviral treatments. There were no significant differences in the mortality and deterioration rate between NGS

and non-NGS group, but it exhibited a trend that the mortality and deterioration rate of NGS group was lower than non-NGS group after the propensity score matching analysis (15.8% vs 24.3%, P=0.173; 25.6% vs 37.8%, P=0.093).

Conclusion: NGS could affect the anti-infection treatments and had a trend of reducing the mortality and deterioration rate of critically ill patients with pneumonia.

KEYWORDS

next-generation sequencing, pneumonia, prognosis, diagnostic effect, ICU

Introduction

Pneumonia, triggered by diverse pathogens like bacteria, virus or fungi, is the most typical infection of patients admitted to the intensive care unit (ICU), with high mortality ranging from approximately 15% to 50% (Li et al., 2016). The source of infection may be one single pathogen, while sometimes it can be accompanied by multiple pathogens. Uncontrolled infection may develop into severe pneumonia, aggravate the inflammation and cause life-threatening organ dysfunction, leading to a poor prognosis (Morris and Medicine, 2018).

Diagnosis and clinical decisions regarding infectious diseases mainly rely on precisely identifying etiologic microorganisms. Bacterial and fungal smear and culture usually work as the gold standard method for identifying causative pathogens. At the same time, polymerase chain reaction (PCR) and antigen tests are currently applied for the detection of viruses. However, traditional pathogen detection methods are time-consuming and inefficient. Empirical antibiotic treatments are often given to patients with negative pathogen results from traditional detection, while the actual pathogens of the infectious source may not be targeted and therefore aggerating the condition of patients (Langelier et al., 2018; Zhang et al., 2021). Early and targeted anti-infection treatments are crucial to reduce pneumonia mortality rate (Xie et al., 2019).

With the rapid development of molecular methods, metagenomic sequencing, also called next-generation sequencing (NGS), emerged as a fast and precise diagnosis of the infection (Behjati and Tarpey, 2013). Compared to the traditional microorganism culture, NGS directly sequence all the nucleic acid fragments in a short time with high-throughput capacity (Gwinn et al., 2019). The results of bioinformatics analyses will precisely illustrate the species of the pathogen within the sample, especially for the rare and slow-growing microorganisms or multiple infections, leading to fast and accurate diagnosis and treatment.

In recent years, the value of NGS has gradually been recognized for infection diagnosis owing to the high efficiency and high positive detection rate in culture-negative samples (Brenner et al., 2018; Tarabichi et al., 2018). However, whether the NGS results can affect medical decisions and consequently improve the prognosis of patients with pneumonia in the ICU has not been reported. The objective of this study is to evaluate the value of NGS in pulmonary infection diagnosis and pathogen identification in ICU patients, by comparing the result of NGS to the conventional detection methods.

Methods

Study design

A retrospective screen was performed from January 2018 to January 2021 for cases of pulmonary infections and pneumonia admitted into the ICU of The Affiliated Hospital of Qingdao University. Patients that had been discharged from hospital before we obtained NGS results were excluded. The specimens of NGS results included blood, bronchoalveolar lavage fluid or sputum. This study was conducted according to the Declaration of Helsinki principles and approved by the Ethics Review Committee of The Affiliated Hospital of Qingdao University (Approval number: QYFYWZLL26515), and was registered on the Chinese Clinical Trial Registry (Registration number: ChiCTR2100050201).

The individual informed consent was waived for this retrospective analysis.

Clinical data collection and antibiotic treatment

Data of targeted patients were initially screened from the registration of discharge and admission, and further information

was collected from the medical records through the Hospital Information System (HIS), including demographics, laboratory test results, APACHE II score, length of mechanical ventilation, length of stay in hospital and ICU, initial antibiotics at ICU admission and adjustment later based on the pathogen results of NGS were also collected. These patients with pneumonia were divided into the NGS group and the non-NGS group by whether they were being performed NGS or not.

Statistical analysis

The t-test was used to determine the normal distribution and uniformity of variance. Also, the student t-test and $\chi 2$ test were used to calculate differences in continuous variables between groups. Propensity score matching (PSM) was performed to more precisely evaluate the diagnostic effects of NGS. Data analyses were performed using the SPSS 26.0 (IBM, Armonk, NY, USA) software. P values < 0.05 were considered significant.

Results

Participants and study design (sample and patient characteristics)

The flow chart of patients included was shown in Figure 1. A total of 641 patients were screened, and 94 patients were excluded based on exclusion criteria. 547 (85.3%) patients were enrolled, among which 160 (29.3%) patients got NGS detection, and 387 (70.7%) patients had conventional detection. The demographic and baseline characteristic of patients enrolled were provided in Table 1. Neither group had significant differences in gender, age and medical history (P >0.01). The APACHE II scores and diabetes ratio were higher in patients who performed NGS (P<0.001). To adjust the imbalanced distribution, propensity score matching (PSM) was performed to more precisely evaluate the diagnostic effects of NGS and conventional methods. NGS (n = 160) patients were 1:1 propensity-matched to non-NGS (n = 387) based on the APACHE II Score.

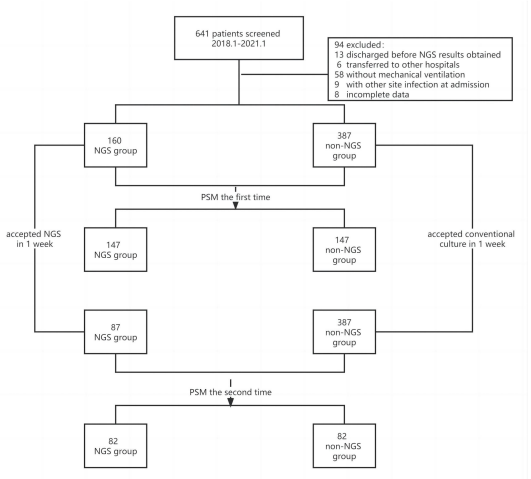

FIGURE 1
Flow chart of patients included.

Clinical impact of NGS-based diagnostics

Among all the patients with pulmonary infections, the percentage of NGS-positive results was 88.75% (142/160), while the positive ratio of conventional methods was 86.25% (138/160). There were no significant differences in the detection rate. However, compared to conventional methods, NGS was more sensitive in detecting pathogens (Figure 2). Among the 142 NGS-positive cases of pulmonary infections, extra pathogens were seen in 132 specimens, including 82 cases with viruses, 18 with fungus, 17 with bacteria, 14 with mycoplasma, and one with *mycobacterium tuberculosis.*

Identification of pathogens in negative conventional pathogen detection specimens by NGS

A total of 31 specimens received negative or non-specific results from conventional pathogen detection tests, in which 23 had positive results and 8 had negative results by NGS. Among the specimens of pulmonary infectious patients enrolled in this study, the substantial pathogens that traditional tests failed to detect included 18 specimens with bacterial, 14 with viruses, six with fungus and two with mycoplasma and *pneumocystis carinii* (Figure 3).

Antibiotic adjustment affected by the diagnostic effect of NGS

Antibiotics were adjusted in patients who performed NGS. Among the 160 patients, 48 (30%) had added or stopped anti-bacterial agents, 20 (12.5%) adjusted antifungal drugs, and 20 (12.5%) adjusted antiviral drugs (Figure 4A). The reduction rate was higher than addition rate in anti-bacterial and antiviral agents (Figure 4B). Although there was no significant difference, it exhibited a trend of purposefully reducing complete pathogen covered empirical antibiotics.

TABLE 1 Baseline characteristics and clinical indices of NGS and non-NGS groups.

	NGS (n=160)	non-NGS (n=387)	P value
Gender (male/female)	114/46	236/151	0.023
Age (years)	63 (53-73)	65 (52-75)	0.282
APACHE II Score	22 (17-26)	17 (12-23)	<0.01
Cardiovascular disease (N/Y)	88/72	176/211	0.043
Cerebrovascular disease (N/Y)	135/25	331/56	0.729
Diabetes (N/Y)	107/53	309/78	0.001
COPD (N/Y)	159/1	372/15	0.048
Cancer (N/Y)	143/17	357/30	0.275
Autoimmune disease (N/Y)	154/6	364/23	0.298
Etiological tests results times(hours)	56.91 ± 8.82	74.96 ± 15.81	<0.01
Duration of mechanical ventilation (d)	13 (6-28)	4 (0-12)	<0.01
Length of stay in ICU (d)	21 (13-42)	12 (5-23)	<0.01
Length of stay in hospital (d)	29 (17-50)	19 (10-35)	<0.01
Mortality rate (%)	20% (32/160)	18.1% (70/387)	0.601
Deterioration rate (%)	33.1% (53/160)	28.7% (111/387)	0.302

Effect of NGS compared with traditional detection methods

After the 1:1 propensity matching, NGS (n = 147) and non-NGS (n = 147) groups were adequately balanced, and 147 cases were included for further analysis (Table 2).

However, some of the patients were performed NGS after an extended stay in the ICU. The time we performed NGS detection was not the actual time of admission to the ICU, the results can be affected by this confounding factor. Consequently, we screened the patients under NGS detection within a week and then performed a matching analysis of 87 cases, 82 cases were matched and included in the final analysis. The baseline analysis is provided in Table 3, and the matching results are provided in Table 4. There was no significance between NGS and non-NGS groups in mortality rate (P=0.173) and deteriorated rate (P=0.093). Compared to Non-NGS patients, the length of stay in ICU and hospital and the duration of mechanical ventilation was longer in NGS patients than in non-NGS patients.

Among the 82 NGS-positive cases after propensity matching, extra pathogens were seen in 61 specimens, including 44 (72.13%) cases with viruses, 9(14.75%) with mycoplasma and 8 (13.11%)

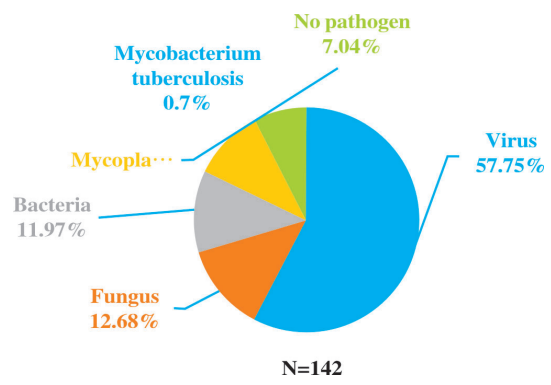

FIGURE 2
Extra pathogens detected by NGS compared to conventional detection methods.

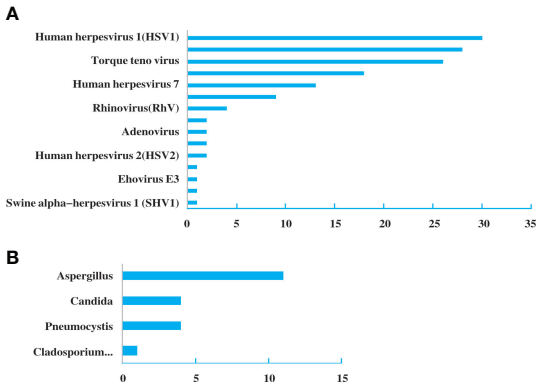

FIGURE 3
Pathogen spectrum of specimens in the NGS group. **(A)**. Bacterial spectrum. **(B)**. Fungal spectrum.

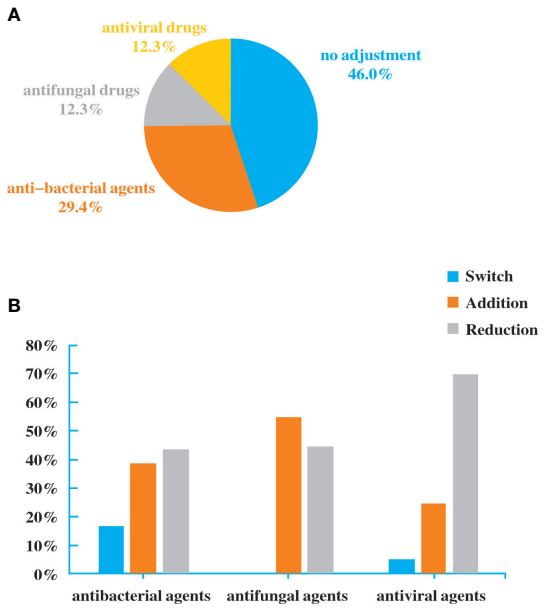

FIGURE 4
Antibiotic adjustment affected by the diagnostic result of NGS. **(A)**. Bacterial spectrum. **(B)**. Fungal spectrum.

with fungus (Figure 5).48 of the patients had their antibiotics adjusted. 26 (54.17%)had adjusted anti-bacterial agents,14 (29.17%) adjusted antiviral drugs and 8 (29.17%)adjusted antifungal drugs (Figure 6).

Discussion

Our retrospective study compared the diagnostic effects of NGS with traditional detection methods, evaluating its impact on antibiotic adjustment and the prognosis of pneumonia patients in the ICU. NGS exhibited fast and comprehensive detection efficacy for mixed pathogens and may potentially prompt more precise antibiotic treatment, thus improving the prognosis of patients with pulmonary infections.

Pneumonia, a commonly occurred respiratory infection, is responsible for approximately 30% of mortality in the patients admitted to ICU (Walden et al., 2014; Rider and Frazee, 2018). While widely being described as bacterial pneumonia, it has spread out all around the world in the recent pandemic with the novel coronavirus (COVID-19), whose mortality rate accounts for 35-50% (Richardson et al., 2020). Pneumonia caused by mixed pathogens is still a significant concern in the ICU. Precise and timely detection of pathogens can optimize antibiotic administration, potentially reverse the progress of the disease, shorten the length of stays and decrease the mortality rate. However, with low concentration levels of the pathogen-associated substances, pathogen detection in the early stage of infection can be non-specific and inefficient, resulting in delayed diagnosis, impeding targeted therapies or causing excessive antibiotic treatment, and eventually leading to poor prognosis (Shen et al., 2020; Zhang et al., 2021). Also, distinguishing some infectious diseases from non-infectious diseases can be really challenging relying only on routine laboratory tests and imaging

TABLE 2 Baseline characteristics and clinical indices of NGS and non-NGS groups after PSM balancing.

	NGS (n=147)	non-NGS (n=147)	P Value
Gender (male/female)	103/44	98/49	0.531
Age (years)	63 (53-73)	65 (51-75)	0.355
APACHE II Score	22 (16-25)	20 (15-26)	0.643
Cardiovascular disease (N/Y)	80/67	83/64	0.725
Cerebrovascular disease (N/Y)	127/20	128/10	0.863
Diabetes (N/Y)	103/44	98/49	0.531
COPD (N/Y)	146/1	147/0	0.9999
Cancer (N/Y)	133/14	135/12	0.681
Autoimmune disease (N/Y)	142/5	142/5	1
Etiological tests results times(hours)	57.10 ± 9.01	77.01 ± 16.98	<0.01
Duration of mechanical ventilation (d)	12 (7-28)	5 (1-15)	<0.01
Length of stay in ICU (d)	21 (12-42)	12 (5-25)	<0.01
Length of stay in hospital (d)	29 (17-50)	19 (10-34)	<0.01
Mortality rate (%)	21.1% (31/147)	25.9% (38/147)	0.335
Deterioration rate (%)	33.3% (49/147)	37.4% (55/147)	0.464

TABLE 3 Baseline characteristics and clinical indices of NGS and non-NGS groups of 87 cases.

	NGS (n=87)	non-NGS (n=387)	P Value
Gender (male/female)	60/27	236/151	0.165
Age (years)	60 (52-72)	65 (52-75)	0.080
APACHE II Score	22 (17-26)	17 (12-23)	<0.01
Cardiovascular disease (N/Y)	47/40	176/211	0.149
Cerebrovascular disease (N/Y)	77/10	331/56	0.469
Diabetes (N/Y)	59/28	309/78	0.015
COPD (N/Y)	87/0	372/15	0.085
Cancer (N/Y)	75/12	357/30	0.073
Autoimmune disease (N/Y)	84/3	364/23	0.445
Etiological tests results times(hours)	57.63 ± 9.22	74.96 ± 15.81	<0.01
Duration of mechanical ventilation (d)	10 (6-21)	4 (0-12)	<0.01
Length of stay in ICU (d)	27 (10-32)	12 (5-23)	<0.01
Length of stay in hospital (d)	21 (12-39)	19 (10-35)	0.124
Mortality rate (%)	17.2% (15/87)	18.1% (70/387)	0.852
Deterioration rate (%)	26.4% (23/87)	28.7% (111/387)	0.674

analysis. Considering patients in the ICU generally combine several underlying diseases, it is more critical to improve the diagnostic efficiency and perform precise treatment (Huang et al., 2020).

Although accurate diagnosis of pulmonary infection is crucial to the improved management and better prognosis, how to precisely detect and identify pathogens remains a big challenge. Conventionally, diagnosis of infection mainly relies

TABLE 4 Characteristics and clinical indices of NGS and non-NGS groups after propensity matching.

	NGS (n=82)	Non-NGS (n=82)	P Value
Gender (male/female)	58/24	54/28	0.502
Age (years)	60 (53-72)	62 (47-72)	0.853
APACHE II Score	22 (17-26)	20 (14-27)	0.356
Cardiovascular disease (N/Y)	44/38	51/31	0.268
Cerebrovascular disease (N/Y)	72/10	78/4	0.094
Diabetes (N/Y)	57/25	59/23	0.731
COPD (N/Y)	82/0	82/0	1
Cancer (N/Y)	73/9	73/9	1
Autoimmune disease (N/Y)	79/3	80/2	0.999
Etiological tests results times(hours)	57.80 ± 8.67	76.05 ± 17.55	
Duration of mechanical ventilation (d)	10 (6-21)	5 (1-14)	0.002
Length of stay in ICU (d)	17 (10-33)	11 (4-23)	0.01
Length of stay in hospital (d)	22 (12-39)	18 (8-31)	0.041
Mortality rate (%)	15.8% (13/82)	24.3% (20/82)	0.173
Deterioration rate (%)	25.6% (21/82)	37.8% (31/82)	0.093

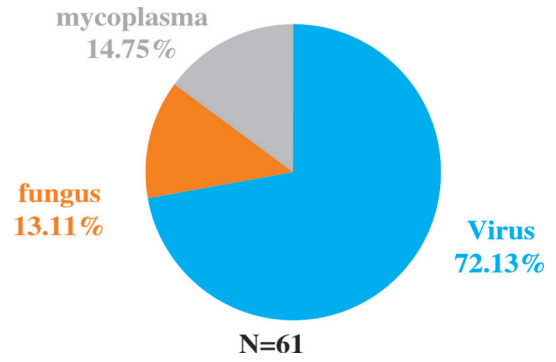

FIGURE 5
Extra pathogens detected by NGS compared to conventional detection methods after propensity matching.

on serological analysis, molecular techniques, histopathology, and smear microscopy (Das et al., 2018; Qian et al., 2020). However, conventional diagnostic practices, like microbial cultures and polymerase chain reaction (PCR), are commonly time-consuming, labor-intensive, and usually lack adequate sensitivity and selectivity (Kumar et al., 2019).

Some precise detection methods have emerged with the urgent need of diagnostic technology, among which the NGS exhibits potential for improving clinical practice and public health through high-throughput technologies and bioinformatics (Gwinn et al., 2017; Gwinn et al., 2019). Our results showed that the percentage with NGS-positive results was 88.75% (142/160). Although there was no significant difference compared to traditional method (86.25%), NGS presented high sensitivity for identifying specific pathogens that are usually not detected in conventional tests. Among the 142 NGS-positive cases of pulmonary infections, 132 specimens had extra pathogens detected, of which 82 cases were virus. *Human herpesvirus* and *Torque teno virus* were the most common pathogens. This might be related to the detection of latent viruses, or it is originated from the reactivation of latent herpesviruses. Studies showed that HSV reactivation is

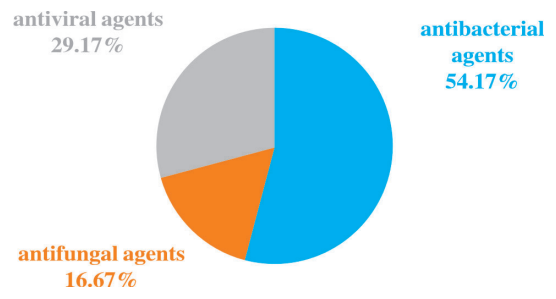

FIGURE 6
Antibiotic Adjustment affected by the diagnosis of NGS after propensity matching.

common in ICU patients and associated with increased morbidity and mortality (Luyt et al., 2020). In addition, HSV reactivation is the initial step toward HSV-associated bronchopneumonitis, which can aggravate lung inflammation or damage and facilitate the occurrence of nosocomial bacterial pneumonia, resulting in prolonged mechanical ventilation. It is an alert that attention should be paid to specific virus infections in critically ill patients with pneumonia. Besides, NGS diagnosis may potentially affect clinical decision. In our study, 48 (30%) patients had anti-bacterial agents added or stopped, 20 had antifungal drugs adjusted and 20 had antiviral drugs adjusted based on the NGS results. The NGS results may contribute to more comprehensively evaluating the empiric antimicrobial therapy and making effective adjustments for critically ill patients with pneumonia in the ICU. The reduction of antibiotics can avert excessive antibiotic treatment and unnecessary healthcare costs while the addition of drugs may target specific pathogens. The mortality and deterioration rate were further analyzed after the 1:1 propensity matching, and a significant difference in APACHE II Score was displayed between the two groups. Considering the time of the admission to the ICU and NGS tests was not the same, we screened the patients that were accepted NGS after being admitted to the ICU within a week, and then completed a matching analysis of 87 cases. 82 cases were matched and included in the final analysis. Although there was still no significant difference between NGS and non-NGS groups in mortality rate (P=0.173) and deterioration rate (P=0.093), it presented a declining trend in the NGS group. We then shortened the difference of time to two days; the matching cases were too few for further analysis. If we enlarge sample size, the two groups' mortality and deterioration rates may present significant differences. Compared to non-NGS patients, the length of stay in ICU and hospital and the duration of mechanical ventilation was longer in NGS patients. The total hospitalization expenses in NGS patients were significantly higher than in non-NGS patients. It is probably because in most ICUs in China, except for specific clinical trials, only when the conditions of patients got worse or the empirical treatment did not work well, the application of NGS were then considered. That is to say, bias obviously existed in the choice of NGS diagnosis, and it may severely affect the results.

In this study, we retrospectively compared the subsequent effects of NGS detection on the prognosis of patients with pneumonia in the ICU and performed propensity score matching based on APACHE II score. The innovative strength of our study is that, by far this is the largest amount of cases analysis on comparing antibiotic management and the prognosis of critically ill patients with pneumonia in the ICU. The clinical data, including demographics, illness history, APACHE II score, length of mechanical ventilation, length of stay in the hospital, length of stay in ICU, and prognosis, were collected and

analyzed. For a more precise analysis of prognosis, we applied propensity score matching to reduce the bias due to confounding variables that the severity of patients' condition may cause. Similarly, in recent articles, Xie et al., reported that the 28-day mortality of the NGS group was significantly lower than the control group (16.7% vs 37.7%, p = 0.008) (Xie et al., 2019). In another study, Zhang. et al. came to similar conclusion that the 28-day mortality rate of the NGS group was significantly lower than that of the non-NGS group (21.4% vs 49.1%, P = 0.006) (Zhang et al., 2020). Consistent with our results, the mortality rate (15.8%) and deterioration rate (24.3%) in NGS group were both lower than in non-NGS group at 25.6% and 37.8% respectively. Although there was no significant difference between the two groups in mortality rate (P = 0.335 to 0.173) and deterioration rate (P = 0.464 to 0.093), it showed the trend that the rate of the NGS group tend to be lower than that in the non-NGS group as we gradually reduced the disturbance of the time length between admission and NGS detection. The NGS detection presented the potential to improve the prognosis of patients with pneumonia.

Our results showed that NGS produced results much faster than traditional methods by about 24 hours or more.At present, our specimens still need to be sent to other places for testing, because the local laboratory is still under construction. Furthermore,NGS is not currently covered by medical insurance in our country. The above resulted in bias in patient selection in this study. If NGS could be tested in hospitals, or if it was cheaper, its advantages might be even greater.

The advantages made NGS a novel, fast, and precise method to better diagnose pulmonary and any other infections of patients. However, conventional detection methods like blood culture are still our first choice. The reasons why NGS has not been served as a routine clinical diagnostic method are quite obvious. Significant barriers out there hinder the further application of NGS in the routine diagnosis of infection: to obtain precise and fast analysis, sophisticated bioinformatics systems, fast data processing and extensive data storage capabilities are required, which drives up the cost. Besides the equipment cost, professional researchers are also highly needed to analyze and clinically interpret the data (Simner et al., 2018). Therefore, it has not been a routine test for the clinic to diagnose infectious diseases and consequently, there is no universal criteria and authoritative guidelines to interpret the report (Zhang et al., 2020).

First, as a retrospective study, the current situation of the NGS application in our hospital mentioned above led to bias in the severity of patients. The application of NGS can even be considered as a process to screen patients ahead of this study. There is no doubt that the condition of patients with NGS detection was worse than that of the non-NGS group, which was precisely reflected in the significant difference between the NGS group and the non-NGS group in the APACHE II score. Second, the time that NGS diagnosis was performed varied a lot. Although we used propensity score matching to reduce the disturbance, the results were still possibly affected by this factor. Also, patients with ventilation and extracorporeal membrane oxygenation (ECMO) were included for analysis. However, the types of pulmonary infections were not specifically distinguished. Finally, our study was also limited by the feature of a single-centred retrospective study. A prospective and multicenter randomized controlled trial is required to comprehensively analyze the essential role of NGS in the whole process of clinical decision-making, including diagnosis, treatment and more importantly, the evaluation of the prognosis of patients.

Conclusion

Our study showed that the accurate and fast detection efficacy enabled NGS to potentially prompt precise antibiotic treatment, improving the prognosis of patients with pulmonary infection in the ICU. The NGS may be a potential substitutable method for the diagnosis of pulmonary infection. However, the complex requirements to perform NGS stalled its application in becoming a routine clinical diagnostic method. For now, it can only be used to assist the clinical diagnosis.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Review Committee of The Affiliated Hospital of Qingdao University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

All authors contributed to the study conception and design. JX contributed to the conception and design of the research; YL and QG collected data and drafted the manuscript; RZ contributed to the design of the research; BY contributed to the literature search and review of manuscript; HG and SZ contributed to the acquisition and analysis of the data. All authors commented on previous versions of the manuscript.

All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of the People's Republic of China (81170179, 81070242 and 81470553).

Acknowledgments

The authors would like to thank Zhihua Lu and WillingMed Technology (Beijing) Co., Ltd for their useful discussions and technical support.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 24 January 2023
DOI 10.3389/fcimb.2022.1083497

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 29 October 2022
ACCEPTED 21 December 2022
PUBLISHED 24 January 2023

CITATION
Wang J, Xu H, Wang X and Lan J (2023) Rapid diagnosis of non-tuberculous mycobacterial pulmonary diseases by metagenomic next-generation sequencing in non-referral hospitals.
Front. Cell. Infect. Microbiol. 12:1083497.
doi: 10.3389/fcimb.2022.1083497

Rapid diagnosis of non-tuberculous mycobacterial pulmonary diseases by metagenomic next-generation sequencing in non-referral hospitals

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Objectives: The incidence of non-tuberculous mycobacterial pulmonary disease (NTM-PD) has increased steadily globally, but the current culture-based diagnosis of NTM-PD is difficult and time-consuming, leading to a high possibility of misdiagnosis. Therefore, new methods should be introduced to improve the processes for clinical diagnosis of this disease.

Methods: Our retrospective observational study enrolled 12 NTM-PD patients who were identified by way of metagenomic next-generation sequencing (mNGS), as well as the characteristic radiological presentation of slowly progressed, usually concomitant bronchiectasis, small cavitary opacity, and multiple nodules that respond poorly to empirical antibiotic therapy. These patients received the recommended drug regimen based on the identified non-tuberculous mycobacteria (NTM) species. Clinical data, including symptoms, laboratory tests, dynamic computed tomography imaging, treatment, and outcome, were recorded and analyzed.

Results: The results of mNGS were all positive, with the standard specifically mapped read numbers (SDSMRN) of NTM ranging from 1 to 766; this was confirmed in six patients via quantitative polymerase chain reaction (qPCR) analysis. The duration fromsample collection tomNGS results was 1–4 days. Among our 12 patients (except for one lost to follow-up) the CT imaging for 11 patients showed significant absorption of lesions.

Conclusions: Our results draw attention to NTM infection as a possible cause of community-acquired pneumonia, especially in patients with suggestive radiological presentation and poor responses to empirical antibiotic therapy. Our study also indicated that mNGS represented a potentially effective tool for the rapid identification of NTM in the respiratory sample. Improved clinician awareness combined with the utilization of mNGS could guide earlier diagnosis and targeted treatment, and finally improved the prognoses of patients with NTM-PD.

KEYWORDS

non-tuberculous mycobacteria (NTM), pulmonary disease, metagenomic next-generation sequencing (mNGS), rapid diagnosis, earlier targeted treatment

Introduction

The incidence of morbidity and mortality owing to non-tuberculous mycobacterial pulmonary diseases (NTM-PD) has increased worldwide and is rapidly becoming a public health problem (Kumar and Loebinger, 2021; Tan et al., 2021). The treatment of non-tuberculous mycobacterial (NTM) pulmonary diseases is particularly challenging as NTM represents over 190 species and subspecies of *Mycobacterium*, other than *M. tuberculosis* and *M. leprae*, and treatment regimens vary for different species (Daley et al., 2020a). Thus, early diagnosis and species detection is very important to guide the clinical targeted treatment of NTM-PD.

Non-tuberculous mycobacteria are ubiquitous in the environment and are generally considered as being of low pathogenicity to humans who have normal immune defenses (Ratnatunga et al., 2020). Increased susceptibility to infection was seen in patients with underlying structural pulmonary diseases, such as cystic fibrosis, chronic obstructive pulmonary disease (COPD), bronchiectasis, or with immunocompromised states (e.g., patients with AIDS, or having had solid organ transplants, or being treated with corticosteroids or other immunosuppressants) (Griffith et al., 2007). In addition, a lower body mass index (BMI) is common among patients with NTM-PD, and is associated with a worse prognosis (Song et al., 2021)

The symptoms of NTM-PD are non-specific and include cough, sputum production, breathlessness, fatigue, weight loss, and fever. NTM infections are typically chronic and slowly progressive. The disease is insidious and it mimics others, which can lead to a delayed diagnosis for patients with NTM-PD. Typical radiographic findings from NTM-PD patients include fibrocavitary, nodules, and bronchiectasis. Patients usually have combined radiological abnormalities, and are subclassified into bronchiectasis with nodules (i.e., "nodular bronchiectatic" phenotype) or cavitation with fibrosis (i.e., "fibrocavitary" phenotype) (Cowman et al., 2019).

The diagnosis of NTM-PD is made based on a combination of clinical, radiographic, and microbiological data (Cowman et al., 2019; Daley et al., 2020a), with the culture of respiratory samples remaining the gold standard for laboratory confirmation of NTM-PD (Daley et al., 2020c). However, the culture-based diagnosis of NTM-PD is difficult and time-consuming. It has been recommended that bacterial culturing is undertaken for at least 8 weeks (Haworth et al., 2017). In addition, the optimal growth temperature differs for NTM species, and as all NTM are fastidious organisms, some, like *M. tilburgii*, have yet to be successfully cultured (van Ingen, 2013). Furthermore, culturing is less applicable with a patients with nodular bronchiectasis because of the low bacterial burden (van Ingen, 2015). Most importantly, because of the lack of clinician awareness of NTM-PD and the poor accessibility to laboratory resources for mycobacterial culture and molecular methods for identification or speciation, the diagnosis of NTM-PD is rarely made in non-referral hospitals. In these settings, unless a predefined suspicious pathogen, a negative culture result of lower respiratory secretions will be reported if growth was not detected within 48 to 72 hours of incubation (York and Gilligan, 2004). Collectively, the culture-based diagnosis of NTM-PD poses great challenges, and the development of alternative, rapid, universally available, and hypothesis-free diagnostic approaches is urgently required.

Metagenomic next-generation sequencing (mNGS) has the potential to detect nearly all pathogens in a clinical sample (Langelier et al., 2018; Chen et al., 2021). Thus, culture-independent and unbiased mNGS has been widely used in investigations of the etiology of infectious diseases (Chiu and Miller, 2019; Gu et al., 2021), and has an obvious advantage in the diagnosis of uncommon respiratory pathogens, including NTM. In addition, the sensitivity of mNGS in NTM identification is higher than that of bacterial culturing (Shi et al., 2020). However, to our knowledge, there have been no studies that systematically evaluate the clinical performance of mNGS in the diagnosis of NTM-PD until now. In this study, we retrospectively investigated the effectiveness of mNGS in identifying NTM-PD. Our study has significant implications for improving clinician awareness and early diagnosis rates of NTM-PD.

Materials and methods

Study design

We conducted a retrospective study of adult inpatients between July 2020 and January 2022 from the Second Clinical Hospital of Chongqing Medical University, a non-referral, tertiary teaching hospital in Chongqing, China. The diagnosis of NTM-PD was made according to the Chinese guidelines for the diagnosis of non-tuberculous mycobacterial diseases in 2021 to 2020 (Chinese Society of Tuberculosis, 2021). According to these guidelines, the patient can be diagnosed with NTM-PD if they meet the required clinical and radiological criteria that cannot be explained by other diagnoses, as well as one of the relevant microbiological criteria. The specific criteria are as follows: (1) pulmonary or systemic symptoms; and (2) nodular or cavitary opacities on a chest radiograph, or a high-resolution computed tomography scan that shows bronchiectasis with multiple small nodules. Patients also had to meet any one of the following microbiological criteria: (A) positive culture results and/or positive NTM molecular biology detection from at least two separate expectorated sputum samples; (B) positive culture results and/or positive NTM molecular biology detection from at least one bronchial wash or lavage; (C) transbronchial or other lung biopsy results with mycobacterial histological features (i.e., granulomatous inflammation or acid-fast bacilli), and positive culture results and/or positive NTM molecular biology detection from a lung biopsy; (D) transbronchial or other lung biopsy results with mycobacterial histological features (i.e., granulomatous inflammation or acid-fast bacilli); and (E) at least one positive culture result and/or positive NTM molecular biology detection from a sputum sample, bronchial wash, or lavage. Based on these guidelines, our criteria for inclusion were as follows: patients were aged between 18 and 80 years, (1) with pulmonary or systemic symptoms, such as fever, fatigue, cough, expectoration; (2) with nodular or cavitary opacities or bronchiectasis with multiple small nodules, as detected on a high-resolution computed tomographic scan, which progress slowly and usually appear concomitantly; (3) who responded poorly to an initial

empiric antibiotic therapy; (4) with positive NTM molecular biology detection from at least one bronchoalveolar lavage fluid (BALF) or sputum sample; and (5) who had a positive response to targeted anti-NTM treatment. Furthermore, we performed fluorescence quantitative polymerase chain reaction (qPCR) analysis using a Applied Biosystems 7,500 real-time PCR system (ABI) to validate the mNGS results. Written informed consent was obtained from all patients or their relatives. This study was approved by the Ethics Committee of the Second Clinical Hospital of Chongqing Medical University [reference number: 2021(104)] and conducted in accordance with the Declaration of Helsinki.

Data collection

Baseline characteristics (including age, sex, underlying samples, such as BALF, sputum specimens, and blood, diseases, medication use history, and epidemiology history), Pneumonia Severity Index (PSI) score on admission, laboratory test results (including routine blood and biochemical tests), imaging findings, and antibiotic treatment, were extracted from electronic medical records. Additional follow-up data on treatments and outcomes were also collected.

Conventional microbiological tests

Routine samples, such as BALF, sputum specimens, and blood, were collected. Conventional microbiological tests were performed on admission, including sputum and BALF culture and smear (acid-fast staining for *M. tuberculosis*), the respiratory pathogen panel by PCR analysis (for the detection of 13 respiratory pathogens), the respiratory pathogen panel by immunological test (for the detection of nine respiratory pathogens), the serum tuberculosis antibody test (TB-Ab IgM, TB-Ab IgG), serum antigen test (1,3-β-D-glucan antigen), the serum and BALF galactomannan test (*Aspergillus* spp.), the serum interferon gamma release assay (*M. tuberculosis*), the tuberculosis skin test (PPD skin test), and the BALF GeneXpert assay (*M. tuberculosis*).

The thirteen respiratory pathogens detected by multiplex PCR were: *Streptococcus pneumoniae*, *Staphylococcus aureus*, meticillin-resistant *Staphylococcus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Haemophilus influenzae*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Legionella pneumophila*, and the *M. tuberculosis complex*. The nine respiratory pathogens detected by immunological testing were: *Legionella pneumophila*, *M. pneumoniae*, *Coxiella burnetii*, *C. pneumoniae*, respiratory syncytial virus, adenovirus, influenza A virus, influenza B virus, and parainfluenza virus.

Metagenomics next-generation sequencing

Samples of BALF (n = 11) and sputum (n = 1) were collected and transported to mNGS laboratories for sequencing following standard procedures (https://emergency.cdc.gov). DNA was extracted using the TIANamp Micro DNA Kit (TIANGEN BIOTECH, Beijing, China) following the manufacturer's instructions. Human DNA was removed using Benzonase (Qiagen) and Tween 20 (Sigma-Aldrich). DNA libraries were constructed through DNA fragmentation, end-repair, adapter-ligation, and PCR amplification using a Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA) or according to the standard protocol of the BGISEQ-500 sequencing platform and Ion Torrent (PGM) platform. The libraries were assessed by an Agilent 2,100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, United States), and quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, MA, United States). Pooled libraries were sequenced on a NextSeq Dx550 sequencer (Illumina, CA, United States), or on a BGISEQ-500 sequencer (BGI, Shenzhen, China), or on an Ion Torrent (PGM) platform (Thermo Fisher Scientific). The sequencing data were trimmed by the removal of low-quality reads and short reads, low-complexity reads, and adapters. The human host sequences were aligned to the human reference genome (GRCh38) using Burrows–Wheeler alignment (BWA). Subsequently, the remaining microbial sequences were classified by simultaneously aligning to four Microbial Genome Databases, consisting of bacteria, viruses, fungi, and parasites. The curated microbial databases were downloaded and optimized from public database, such as Biotechnology Information Reference Sequence (RefSeq) database (release version 68).

Criteria for a positive mNGS result for non-tuberculous mycobacteria

The species-specific read number (SSRN) was also described as specifically mapped read number (SMRN). In this study, SMRN was normalized to 20 million (M) of the total number of sequencing reads, which was called 'standard SMRN' (SDSMRN). The equation for the SDSMRN was as follows: SDSMRN = SMRN × 20 million/total sequencing reads. Non-tuberculous mycobacteria were considered detected if: (1) its genus was among the top 20 with the highest SDSMRN; (2) it was ranked first within its genus; and (3) it had a SDSMRN of > 1 (Qian et al., 2020).

qPCR detection of non-tuberculous mycobacteria

To validate the mNGS results, we performed fluorescence quantitative polymerase chain reaction (qPCR) analysis on an Applied Biosystems 7,500 real-time PCR system (ABI). Table 1 primer sequences used for qPCR analysis of selected NTM species.

Results

Clinical *features and laboratory examinations* of the participants

A total of 1362 patients in our hospital received a mNGS analysis. A total of 12 (0.88%) patients, who were suggestively diagnosed with NTM-PD as a result of mNGS analysis, were retrospectively evaluated in our study. The demographic and clinical characteristics of these patients are shown in Table 2. Among them, five were male and seven were female, with a median age of 60 years (age ranged from 26 to 75

TABLE 1 The amplification primers for NTM are listed below:

Species of NTM	Forward primer (5′–3′)	Reverse primer (5′–3′)
M. kansasii	GCGGAGCTTGCCTATACATTTG	AGGTCCGTACAGTTCCATCTC
M. avium	AAATGCGCTCGTACCGAATC	CCGTACAGCAGGATCAACAC
M. intracellulare	GTTGGCGATCACTCGCTACT	CCGAACCCATCCACGTTTCC
M. abscessus	TGTCGTCTGTGCATCAACTC	CCAATGCTGGACCCAAACAC
M. fortuitum	AGCCAGCCACCACGAATATC	GGCTGAGGAACTCCGAACAC

years; interquartile range 34.75–66.75 years) and body mass index (BMI) of 19.96 kg/m^2 (interquartile range 18.50–21.53 kg/m^2). In total, eight patients had at least one underlying illness (e.g., COPD or coronary heart disease), while only one patient (i.e., patient 2) had a history of bronchiectasis. The duration of illness from onset to admission varied largely, ranging from 1 day to over 3 years, with a median of 20 days. The usual respiratory symptoms of these patients were coughing, sputum production, and dyspnea. Systemic symptoms, such as fatigue, chills, and low-grade fever, were also common. One patient presented with symptoms associated with the location of lesion (i.e., patient 8). The PSI score was calculated for 12 patients, with a medium score of 50.5 (interquartile range, 26–60.25), and only one patient was scored in risk class 4.

As shown in Table 3, on admission, patients 5 and 7 had decreased absolute counts and percentages of lymphocytes, patients 2 and 4 had slightly increased absolute counts and percentages of neutrophils, patients 5 and 10 had slightly increased levels of C-reactive protein (CRP) and hypersensitive C-reactive proteins, and four patients had an increased erythrocyte sedimentation rate (ESR). Procalcitonin (PCT) levels and white blood cell count (WBC) was slightly increased in two patients. The levels of total protein and albumin decreased in patients 5 and 6, respectively. Levels of serum creatinine (CR) decreased in six patients and levels of blood urea nitrogen (BUN) increased in three patients. Except for patient 11, who had increased levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) induced by anti-tuberculosis drugs,

TABLE 2 Baseline characteristics, clinical manifestation, and radiographic findings of patients on admission.

Characteristics on admission	Patients	Normal range
Male sex, *n* (%)	5/12 (41.67%)	
Age (years), median value (range)	60 (34.75–66.75)	
BMI (kg/m^2), median value (range)	19.96 (18.50–21.53)	18.5–23.9
Underlying conditions, *n* (%)		
Bronchiectasis	1/12 (8.33%)	
Asthma	1/12 (8.33%)	
COPD	3/12 (25%)	
Diabetes mellitus	1/12 (8.33%)	
Hypertension	2/12 (16.67%)	
Coronary heart disease	3/12 (25%)	
Dermatomyositis	1/12 (8.33%)	
Duration from illness onset to admission (days), median value (range)	20 (14.50–60)	
Currently a smoker, *n* (%)	4/12 (33.33%)	
Currently an alcoholic, *n* (%)	3/12 (25%)	
Clinical manifestations, *n* (%)		
Fever > 38.5°C	1/12 (8.33%)	
Cough	6/12 (50%)	
Expectoration	6/12 (50%)	
Hemoptysis	2/12 (16.67%)	
Dyspnea	4/12 (33.33%)	
Palpitation	1/12 (8.33%)	

(Continued)

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TABLE 2 Continued

Characteristics on admission	Patients	Normal range
Shiver	1/12 (8.33%)	
Fatigue	1/12 (8.33%)	
Chest tightness	1/12 (8.33%)	
Abdominal pain	1/12 (8.33%)	
Chest pain	1/12 (8.33%)	
Weight loss	1/12 (8.33%)	
Heart rate (beats per min), median value (range)	90 (83.75–100)	60–100
Respiration (breaths per min), median value (range)	20 (20–20.25)	12–20
Pneumonia Severity Index (PSI)		
Median (interquartile range)	50.5 (26–60.25)	
Risk class, *n* (%)		
1–3	11/12 (91.67%)	
4	1/12 (8.33%)	

COPD, chronic obstructive pulmonary disease.

TABLE 3 Laboratory findings on admission.

Characteristics	Patients, *n* (%)	Normal range	Unit
Routine blood test			
White blood cell count (> 9.5)	2/12 (16.67%)	3.5–9.5	10^9/L
Neutrophil count (> 6.3)	2/12 (16.67%)	1.8–6.3	10^9/L
Neutrophil percentage (> 75)	4/12 (33.33%)	40–75	%
Lymphocyte count(< 1.1)	5/12 (41.67%)	1.1–3.2	10^9/L
Lymphocyte percentage (< 20)	7/12 (58.33%)	20–50	%
Monocyte count (> 0.6)	3/12 (25%)	0.1–0.6	10^9/L
Monocyte percentage (> 10)	3/12 (25%)	3–10	%
Number of red blood cells(> 5.8 or < 4.3)	2/12 (16.67%)	4.3–5.8	10^{12}/L
Hemoglobin (> 150 or < 115)	2/12 (16.67%)	115–150	g/L
Platelet distribution width (< 15.5)	7/12 (58.33%)	15.5–18.0	fL
Thrombocytocrit (< 0.11)	3/12 (25%)	0.11–0.28	%
Blood biochemistry			
Erythrocyte sedimentation rate (> 20)	5/12 (41.67%)	0–20	mm/h
Fibrinogen (> 3.5)	4/12 (33.33%)	1.8–3.5	g/L
Prothrombin time (> 14.5)	3/12 (25%)	11–14.5	s
Activated partial thromboplastin time (> 43.5)	3/12 (25%)	31.5–43.5	s
Blood potassium (> 5.1 or < 3.5)	1/12 (8.33%)	3.5–5.1	mmol/L
Blood sodium (< 137)	3/12 (25%)	137–145	mmol/L
Blood chlorine (< 99)	2/12 (16.67%)	99–110	mmol/L
Blood calcium (> 2.92 or < 2.12)	3/12 (25%)	2.12–2.92	mmol/L

(Continued)

TABLE 3 Continued

Characteristics	Patients, *n* (%)	Normal range	Unit
Total protein (< 65)	5/12 (41.67%)	65–85	g/L
Albumin (< 40)	6/12 (50%)	40–55	g/L
Total bilirubin (> 28 or < 5.1)	1/12 (8.33%)	5.1–28	μmol/L
Gamma-glutamyltransferase (> 28)	2/12 (16.67%)	7–45	U/L
Alanine transaminase (> 40)	1/12 (8.33%)	7–40	U/L
Aspartate aminotransferase (> 40 or < 15)	2/12 (16.67%)	15–40	U/L
Lactate dehydrogenase (> 250 or < 120)	1/12 (8.33%)	120–250	U/L
Lactate (> 3.96 or < 1.32)	1/12 (8.33%)	1.32–3.96	mmol/L
Creatine kinase (< 38)	2/10 (25%)	38–174	IU/L
Creatinine (< 62)	6/12 (50%)	62–102	μmol/L
Blood urea nitrogen (> 6.1)	3/12 (37.5%)	2.5–6.1	umol/L
Pro-B-type natriuretic peptide (> 125)	4/12 (50%)	0.00–125.00	pg/ml
Blood glucose (> 5.9)	3/12 (43%)	4.1–5.9	mmol/L
Tissue polypeptide antigen (> 75)	5/11 (0.5)	0–75	U/L
Immunoglobulin E (> 100)	3/9 (42.86%)	0–100	IU/mL
C-reactive protein (≥ 10)	5/12 (36.36%)	< 10	mg/L
Hypersensitive C-reactive protein (≥ 1)	10/12 (81.82%)	< 1	mg/L
Procalcitonin (> 0.05)	2/11 (20%)	0–0.05	ng/mL
T-cell subpopulation			
$CD3^+$ T-cell counts (< 770)	5/8 (57%)	770–2860	/μL
$CD3^+CD4^+$ T cells (< 500)	7/8 (86%)	500–1440	/μL
$CD3^+CD8^+$ T cells (< 238)	4/8 (43%)	238–1250	/μL
CD4/CD8(> 2.47 or < 1.00)	1/8 (14%)	1.00–2.47	

IU, international unit.

all patients had normal ALT and AST levels. $CD3^+$, $CD4^+$, and $CD8^+$ T-cell counts were available for eight patients and patients 4, 5, and 7 had reduced $CD3^+$, $CD4^+$, and $CD8^+$ T-cell counts, respectively.

Radiological features of the participants

Consistent with previous reports, multiple nodules (found in 11 out of 12 patients), bronchiectasis (found in 10 out of 12 patients) and cavitary opacities (found in 6 out of 12 patients) (Supplement Figure 1) were the radiologic features most patients had in common were the major patterns found. In addition, pulmonary consolidation was present in two patients (i.e., patients 2 and 8; Supplement Figure 1G and Supplement Figure 1J). The bronchiectasis was usually mild or moderate, multifocal, within the focal lesion correlated with localized fibrosis traction, and with no obvious peripheral exudation except for in patient 2, who had a history of bronchiectasis in which the lesion was diffuse and extensive. Each lobe, in each lung, could be involved. All the bronchiectasis were cylindrical, and mucus plugging was seen in 6 out of 10 bronchiectasis patients. After anti-NTM treatment, resolution of mucus plugging was found, but bronchiectasis was not ameliorated.

The infectious species found were *M. kansasii* (4), *M. abscessus* (3), *M. intracellulare* (2), *M. fortuitum* (2), and *M. avium* (1). The cavitary opacity is usually localized in the upper lobes, and the cavity is generally small with a thick wall or even occasionally with an air–fluid level. The species which most often had a CT presentation of cavitary opacity was *M. kansasii*. Among the six patients presenting with cavitary opacity, three were infected by *M. kansasii*, one was infected by *M. avium*, one by *M. intracellulare*, and one by *M. abscessus*. The multiple nodules were characterized with centrilobular distribution, occasionally with a tree-in-bud appearance, which appeared with or without the concomitant presence of bronchiectasis or cavity. The infectious species were *M. kansasii* (by which four patients were infected), *M. abscessus* (by which three patients were infected), *M. avium* (by which one patient were infected), *M. intracellulare* (by which two patients were infected), and *M. fortuitum* (by which two patients were infected). Usually, CT appearances of bronchiectasis, cavity and multiple nodules presented simultaneously, progressed slowly, and absorbed slowly usually with residual lung lesions after treatment.

Conventional *microbiological tests results*

On admission, routine microbiology tests for common pathogens of community-acquired pneumonia (CAP) and *M. tuberculosis* (MTB), including serology, sputum smear and culture, and blood culture, were performed. Only patient 12's sputum culture had the positive result for the test indicating the presence of *P. aeruginosa*. None of the patients had a positive result for the sputum smear test.

Among the 10 patients who took the serum antibody test for MTB, only one tested positive for the presence of immunoglobin M (IgM), and three tested positive for the presence of immunoglobulin G (IgG). None of the patients had a positive result for the BALF Xpert assay. Among the 12 patients who took the purified protein derivative test (PPD) for tuberculosis, patients 1, 4, and 5 were mildly (+), moderately (++) and severely (+++) positive, respectively. Among the 10 patients who took the interferon gamma release assay (IGRA), four patients were positive. None of the patients had a positive result for the serum (1–3)-β-D-glucan (G test) assay, or for the serum and BALF galactomannan (GM) assay (Table 4).

As conventional microbiological testing could not provide valuable clues for the diagnosis, and the adequate duration of empiric antibiotic therapy was ineffective, BALF (11 patients) and sputum (patient 7, who had a history of anesthesia-related allergy) samples were collected for mNGS.

The SDSMRN of non-tuberculous mycobacteria were detected in all samples. Based on Chinese guidelines, and the interpretation of mNGS results, six patients were diagnosed with NTM pulmonary diseases by BALF mNGS; these patients comprised one who had been infected by *M. avium*, one infected by *M. intracellulare*, one infected by *M. kansasii*, two infected by *M. fortuitum*, and one infected by *M. abscessus*. The SDSMRN of non-tuberculous mycobacteria, with which raw sequencing data were normalized to 20M reads, ranged from 19 to 766. Another 6 patients were diagnosed as suspected NTM pulmonary diseases due to the small number of nontuberculous mycobacteria reads (lower than 10 SDSMRN) with medium confidence. Another 6 patients were diagnosed as suspected NTM pulmonary diseases due to the small number of nontuberculous mycobacteria reads (lower than 10 SDSMRN) with medium confidence. Among them, the small unique reads of *M. intracellulare* (BALF), *M. abscessus* (*sputum*) and *M. kansasii* (BALF) were found in 1, 2 and 3 patients, respectively. qPCR was subsequently used to validate the results of mNGS in nine patients (Figures 1, 2; Figures S2–8). Six BALF samples from patients 2, 3, 4, 8, 10, and 12 were detected to be positive (Figure 2; Figures S3, S4, S6, and S7). Interestingly, the qPCR result of the BALF sample from patient 8 was positive, with four SDSMRNs of the *M. fortuitum* detected by an mNGS test (Figure 2). The mNGS and qPCR results are shown in Table 5.

Treatment and *outcome*

On admission, all patients, except patient 11, were treated with β-lactam/β-lactamase inhibitor combinations and/or quinolones. Patient 11 was given isoniazid, rifampicin, ethambutol, and pyrazinamide because they had been diagnosed with pulmonary tuberculosis at a local hospital. When mNGS results were obtained, their treatments were adjusted to antimicrobial therapy for NTM-PD according to the ATS/ERS/ESCMID/IDSA guideline (Daley et al., 2020b) (Table 6). All

TABLE 4 Conventional microbiological tests.

No.	Culture	Sputum Smear	The respiratory pathogen panel by PCR	The respiratory pathogen panel by immunological test	G test	GM test	Xpert MTB/RIF	TB-Ab IgM	TB-Ab IgG	PPD[a]	IGRA
P1	Negative	Negative	NA	NA	NA	NA	NA	NA	NA	+	NA
P2	BALF: *Actinomyces dentocariosus*	Negative	NA	NA	Neg	Neg	Negative	Negative	Negative	+	+
P3	Negative	Negative	NA	NA	Neg	Neg	Negative	Negative	Negative	Negative	+
P4	Negative	Negative	NA	NA	Neg	Neg	Negative	Negative	+	++	Negative
P5	Negative	Negative	NA	NA	Neg	Neg	Negative	Negative	Negative	++	+
P6	Negative	Negative	NA	NA	Neg	Neg	Negative	Negative	Negative	+	Negative
P7	Negative	Negative	NA	NA	Neg	Neg	NA	Negative	Negative	++	Negative
P8	Negative	Negative	NA	NA	Neg	Neg	Negative	Negative	Negative	+	Negative
P9	Negative	Negative	Negative	Negative	Neg	Neg	Negative	+	+	+	+
P10	Sputum: *Pseudomonas aeruginosa*	Negative	Negative	Negative	Neg	Neg	Negative	Negative	Negative	++	Negative
P11	Negative	Negative	Negative	Negative	Neg	Neg	Negative	Negative	Negative	+++	Negative
P12	Negative	Negative	Negative	Negative	Neg	Neg	Negative	NA	NA	Negative	Negative

G test, 1,3-β-D-glucan test; GM test, galactomannan test; TB-Ab IgM, human tuberculosis antibody IgM test; TB-Ab IgG, human tuberculosis antibody IgG test; PPD, purified protein derivative test for tuberculosis; IGRA, interferon-gamma release Assays.
a: Reaction was observed between 48-72 hours for maximum induration size and results were interpreted as: 0-5 mm negative, 5-10 mm mildly positive +, 10-20 mm moderately positive ++, more than 20 mm strongly positive +++.

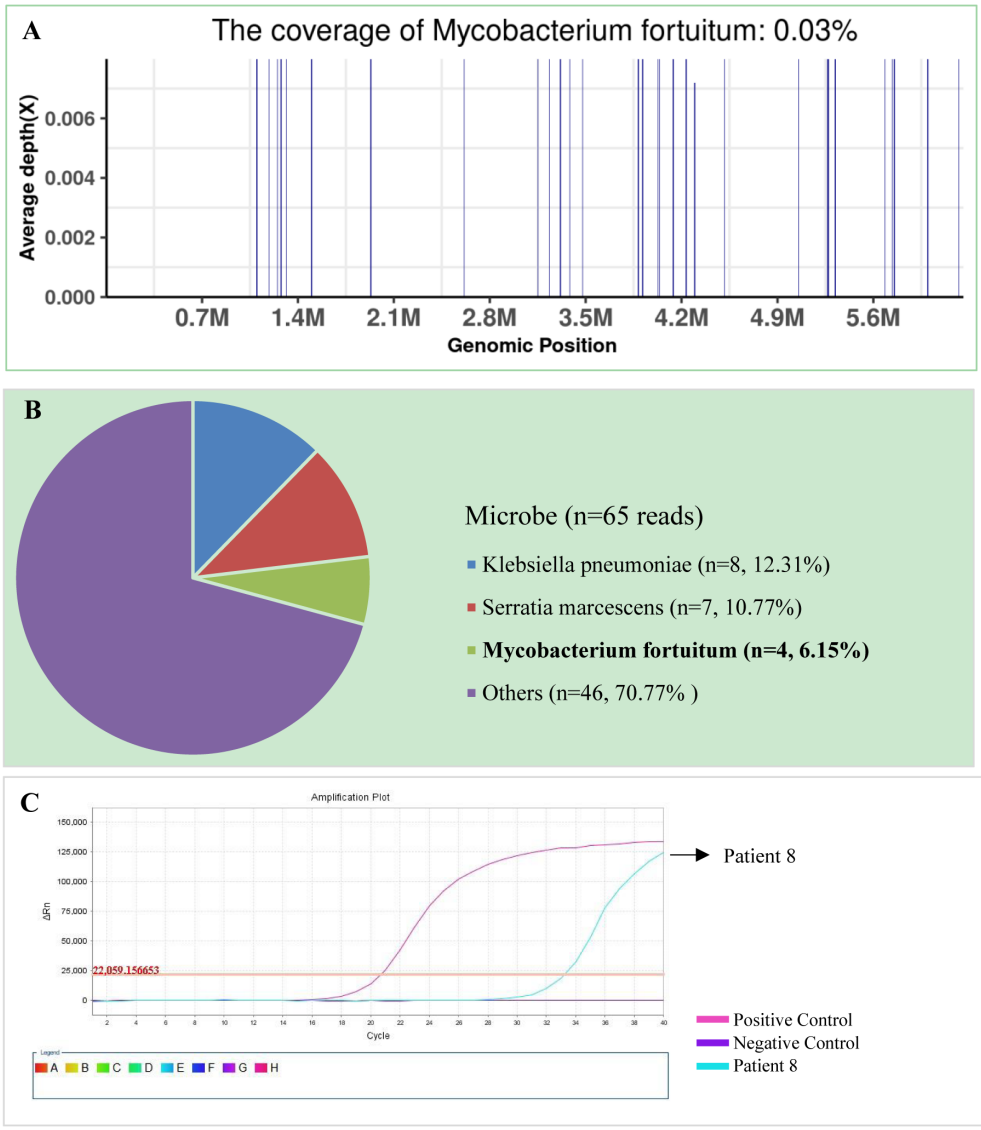

FIGURE 1
The bronchoalveolar lavage fluid (BALF), metagenomic next-generation sequencing (mNGS), and quantitative polymerase chain reaction (PCR) results for patient 8. **(A)** The genome coverage of *Mycobacterium fortuitum*, with 30 reads mapped to the genome of *M. fortuitum*. **(B)** The species composition of the BALF microbes, with four species-specific reads mapped to the genome of *M. fortuitum*. **(C)** Positive qPCR results for *M. fortuitum*.

patients, except patient 12, were discharged 1-11 days after anti-NTM therapy, with their symptoms either alleviated significantly or diminishing. Obviously, for patient 12, her symptoms were also alleviated, but she was discharged 2 months after receiving anti-NTM therapy because of her dermatomyositis. As treatments for NTM-PD must be administered for prolonged periods, all patients, except patient 10, are still in the follow-up phase. Until now (after about 2–3 months of anti-NTM treatment), among the remaining 11 patients, the lesions detectable on CT imaging show significant absorption (Figure 3).

Discussion

Our retrospective study is the first to systematically evaluate the clinical performance and effectiveness of mNGS in the diagnosis of NTM-PD out of its referral institution. In our study, 12 patients who responded poorly to initial empiric antibiotic therapy were diagnosed with NTM-PD by way of mNGS, and given the recommended drug based on the isolated bacterial species by which they were infected. Symptoms disappeared or were subsequently alleviated in all the patients. In addition, CT imaging showed significant absorption of the lesions. In our study, the turnaround time of mNGS detection ranged from 1 day to 4 days, and was therefore significantly shorter than the turnaround time for the traditional NTM culturing process. Our result indicates that mNGS testing is an effective tool for the rapid identification of NTM. This approach could guide earlier and more targeted treatment and, finally, improve the prognoses of patients with NTM-PD.

According to the ATS/ERS/ESCMID/IDSA clinical practice guidelines for the treatment of non-tuberculous mycobacterial pulmonary disease in 2020 (Daley et al., 2020a), the main microbiological evidence of NTM pulmonary disease is positive

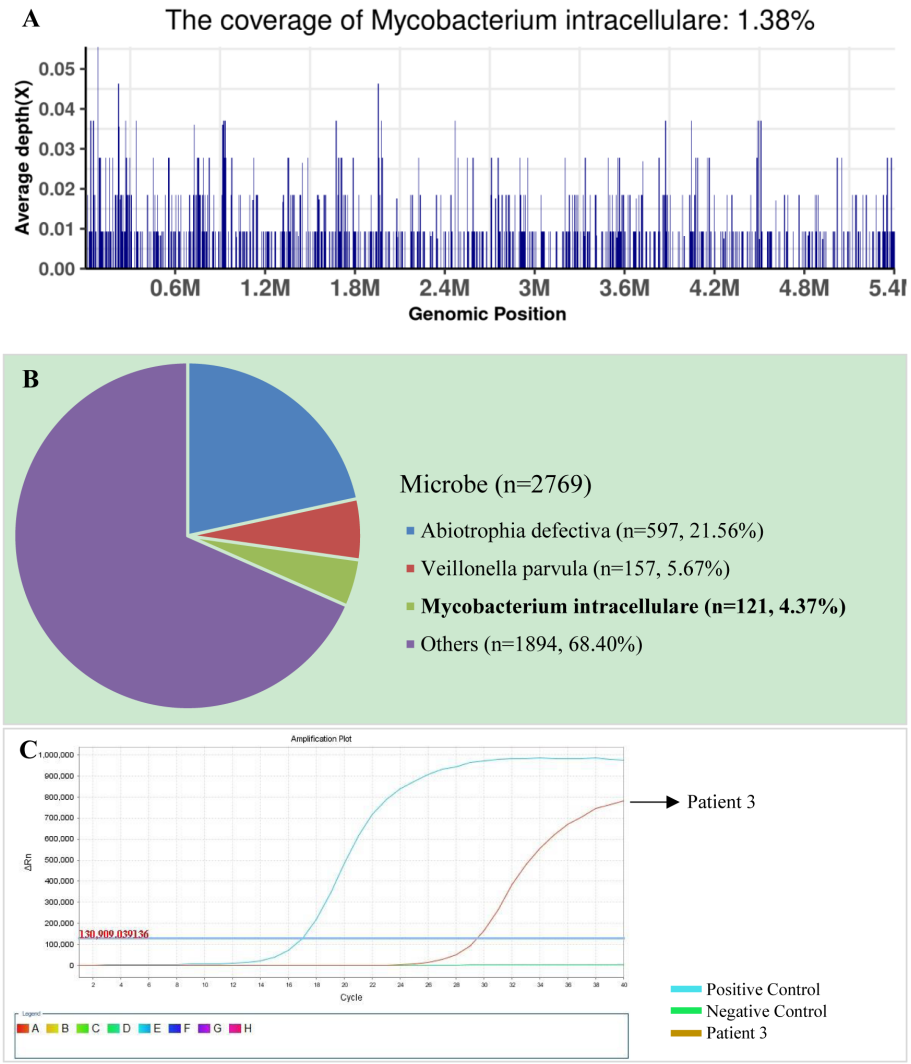

FIGURE 2
The bronchoalveolar lavage fluid (BALF), metagenomic next-generation sequencing (mNGS), and quantitative polymerase chain reaction (PCR) results for patient 3. **(A)** The genome coverage of *Mycobacterium intracellulare*, with 812 reads mapped to the genome of *M. intracellulare*. **(B)** The species composition of the BALF microbes, with 121 species-specific reads mapped to the genome of *M. intracellulare*. **(C)** Positive qPCR results for *M. intracellulare*.

culture results from respiratory samples or positive acid-fast staining smears or granulomatous inflammation from lung biopsies. Released in the same year, but a little later than the ATS/ERS/ESCMID/IDSA clinical practice guidelines, the Chinese guidelines added nucleic acid detection from bronchial washes or lavage as microbiological evidence of the presence of NTM (Chinese Society of Tuberculosis, 2021). Mycobacterial culturing was not routinely performed in our non-referral hospitals, both because it is time-consuming of mycobacterial culture and because of poor clinician awareness of NTM-PD. Therefore, we chose the Chinese guidelines to diagnose NTM pulmonary disease, and six patients were definitively diagnosed with NTM-PD. Owing to the difficulty of DNA extraction for NTM, we considered the mNGS result as positive when SDSMRN was ≥ 1. Meanwhile, because NTM may be due to environmental or hospital contamination, we considered a SDSMRN of ≥ 10 as the credible value for the NTM infection; this was based on a consensus in the suggestions of Chinese experts (Chinese Society of Bacterial Infection and Drug Resistance Prevention, 2022). Considering clinical manifestations, radiological imaging, and good response to anti-NTM treatment in combination, we regarded patients as having a confirmed case of NTM pulmonary disease when the SDSMRN was ≥ 10 (Chinese Society of Bacterial Infection and Drug Resistance Prevention, 2022) and as suspected NTM pulmonary disease when the SDSMRN was ≥ 1.

Several studies have validated the diagnostic performance of mNGS in the NTM pulmonary diseases. 281 One study (Xu et al., 2022) stated that mNGS and acid-fast staining was positive in 23 and 6 NTM 282 samples, showing 100% and 26.08% sensitivity, separately. In our study, all 12 NTM samples can be identified by using mNGS with 100% sensitivity, too. However, all acid-fast staining results were negative with 0% sensitivity. Another study (Wei et al., 2022) compared the performance of mNGS and Bactec mycobacterial growth indicator tubes (MGIT) 960 in NTM-PD diagnosis and found that the sensitivity of mNGS in NTM-PD diagnosis was 81.4%. The sensitivity of MGIT 906 in NTM-PD diagnosis was 53.6%, and the mNGS and MGIT 960 used in

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TABLE 5 mNGS and PCR results.

Patient	Sample	Platform	NTM	SDSMRN	Other pathogens detected by mNGS (SSRN)	Turnaround time of mNGS detection	Time point of NGS test after admission	qPCR results
Patient 1	BALF	NextSeq 550Dx sequencing platform	*Mycobacterium kansasii*	1	Human betaherpesvirus *7* (11)	2 days	3 days	Negative
Patient 2	BALF	NextSeq 550Dx sequencing platform	*Mycobacterium avium*	119	None	2 days	18 days	Positive
Patient 3	BALF	NextSeq 550Dx sequencing platform	*Mycobacterium intracellulare*	104	*Tropheryma whipplei* (11)	2 days	6 days	Positive
Patient 4	BALF	NextSeq 550Dx sequencing platform	*Mycobacterium intracellulare*	5	*Enterobacter cloacae* complex (5)	2 days	1 day	Positive
Patient 5	BALF	NextSeq 550Dx sequencing platform	*Mycobacterium kansasii*	3	Human betaherpesvirus 4 (32)	2 days	15 days	Negative
Patient 6	BALF	Ion Torrent proton sequencer	*Mycobacterium kansasii*	542	*Rhinovirus A* (235871)	3 days	2 days	None
Patient 7	Sputum	NextSeq 550Dx sequencing platform	*Mycobacterium abscessus*	1	*Streptococcus pneumoniae* (6339); *Haemophilus influenza* (331); human betaherpesvirus 4 (288); and human betaherpesvirus 7 (56)	3 days	9 days	None
Patient 8	BALF	NextSeq 550Dx sequencing platform	*Mycobacterium fortuitum*	19	None	4 days	16 days	Positive
Patient 9	BALF	BGISeq-50/ MGISeq-2000 platform	*Mycobacterium kansasii*	1	*Enterococcus faecium* (11); human betaherpesvirus 7 (1); and *Schizophyllum commune* (6)	4 days	4 days	None
Patient 10	BALF	NextSeq 550Dx sequencing platform	*Mycobacterium abscessus*	766	*Pseudomonas aeruginosa* (5580); and *Candida albicans* (4)	1 day	5 days	Positive
Patient 11	BALF	NextSeq 550Dx sequencing platform	*Mycobacterium abscessus*	5	Human betaherpesvirus 4 (3)	1 day	8 days	Negative
Patient 12	BALF	NextSeq 550Dx sequencing platform	*Mycobacterium fortuitum*	37	*Aspergillus flavus* (11); and *Pneumocystis jirovecii* (13)	1 day	6 days	Positive

BALF, bronchoalveolar lavage fluid.

combination in NTM-PD diagnosis was 91.8%. Above all, these results indicate that the performance of mNGS in the diagnosis of NTM-PD was much better than acid-fast staining and bacterial culturing, but a little lower than the combined methods. Therefore, mNGS may be used complementarily with acid-fast staining and bacterial culturing in the diagnosis of NTM-PD, and this combined use of mNGS, acid-fast staining, and bacterial culturing may largely improve the diagnostic efficiency in the future. Besides NTM, mNGS also detected the presence of other common pathogens causing respiratory infections, such as *P. aeruginosa* in patient 10, the presence of which was confirmed in the sputum sample.

Differentiation between NTM infection and tuberculosis (TB) is important but difficult. Although recognized radiological presentations of bronchiectasis, cavitary opacities, and multiple nodules are common

TABLE 6 Treatment and outcomes.

Patient	Antimicrobial therapy before NTM diagnosis	Antimicrobial therapy after NTM diagnosis	Outcome
Patient 1	Piperacillin/tazobactam	Isoniazid + ethambutol + rifapentine	Symptoms disappeared
Patient 2	Piperacillin/tazobactam; then moxifloxacin + meropenem	Azithromycin + moxifloxacin	Symptoms alleviated obviously
Patient 3	Piperacillin/tazobactam + levofloxacin	Azithromycin + rifampicin + ethambutol	Symptoms alleviated obviously
Patient 4	Levofloxacin	Azithromycin + rifampicin + ethambutol	Symptoms alleviated
Patient 5	Piperacillin/tazobactam	Isoniazid + rifampicin + ethambutol	Symptoms alleviated obviously
Patient 6	Moxifloxacin	Isoniazid + rifampicin + ethambutol	Symptoms disappeared
Patient 7	Biapenem	Azithromycin + faropenem + amikacin	Symptoms alleviated obviously
Patient 8	Piperacillin/tazobactam + moxifloxacin	Moxifloxacin + minocycline + sulfamethoxazole	Symptoms disappeared
Patient 9	Levofloxacin	Isoniazid + rifampicin + ethambutol	Symptoms alleviated obviously
Patient 10	Piperacillin/tazobactam + moxifloxacin	Azithromycin + faropenem + amikacin	Symptoms disappeared
Patient 11	Isoniazid + rifampicin + ethambutol + pyrazinamide	Azithromycin + faropenem + amikacin	Symptoms alleviated obviously
Patient 12	Cephalosporins + moxifloxacin	Sulfamethoxazole + voriconazole + moxifloxacin	Symptoms alleviated obviously

indicators of NTM-PD, it is difficult to diagnose NTM-PD based solely on these clinical manifestations and imaging findings, since considerable radiological overlap exists between NTM-PD and pulmonary tuberculosis (PTB) (Musaddaq and Cleverley, 2020). Usually, the diagnosis of NTM-PD is rare in the majority of hospitals without mycobacterial reference laboratories, owing to the lack of clinician awareness and poor access to adequate laboratory resources for NTM species identification or speciation (Sharma and Upadhyay, 2020), especially in high-TB-burden countries and areas, where acid-fast bacilli sputum smear tests are the primary method for the diagnosis of PTB (Gopinath and Singh, 2010). There is a high possibility of misdiagnosis of NTM-PD, as PTB diagnosis using anti-acid staining

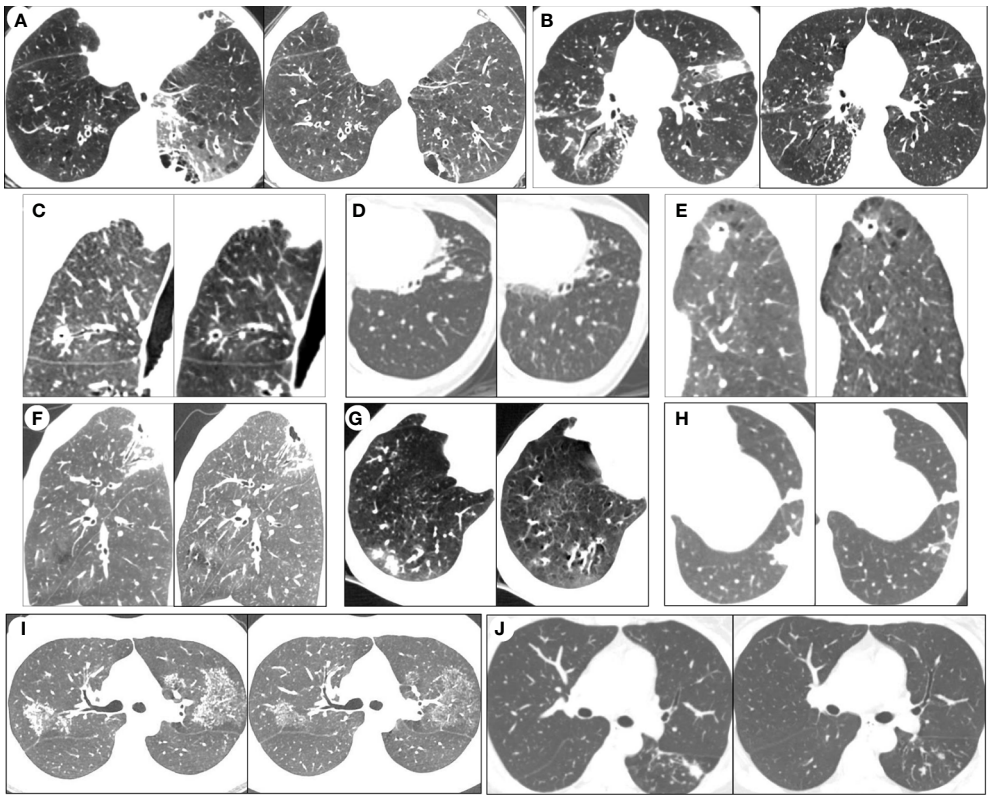

FIGURE 3
Serial chest computed tomography (CT) scans of selected patients with NTM-PD before and after the anti-NTM treatment. After 2 to 3 months of anti-NTM treatment, HRCT imaging showed significant absorption of lesions in patient 1 **(A)**, patient 2 **(B)**, patient 3 **(C)**, patient 4 **(D)**, patient 5 **(E)**, patient 6 **(F)**, patient 7 **(G)**, 464 patient 8 **(H)**, patient 9 **(I)** and patient 11 **(J)**.

smear tests is not able to differentiate NTM from the *M. tuberculosis* complex, and these patients are empirically treated with anti-TB drugs (Gopalaswamy et al., 2020). Usually, NTM species are resistant to first-line anti-TB drugs, thus, this misdiagnosis will result in inappropriately prolonged, and potentially toxic treatment and poor outcomes (Shao et al., 2015). In our study, 10 out of 12 patients and 4 out of 10 patients were positive for the PPD test and IGRA, respectively, which are important methods used in the diagnosis of TB. It has been reported that the accuracy measures of PPD are often confounded by the bacillus Calmette–Guérin (BCG) vaccination and non-tuberculous mycobacterial (NTM) infections (Sharma et al., 2017). Although it has been claimed that IGRA is more specific than PPD in the diagnosis of TB (Ji et al., 2017), a few NTM species like *M. kansasii* could also induce a positive result for IGRA (Jagielski et al., 2019); in our study, two of the four patients with positive IGRAs were infected by *M. kansasii*. In addition, it has been reported recently that IGRA possesses limited discriminatory power for the detection of NTM in AFB smear-negative patients (Yang et al., 2020). Finally, we could not exclude the possibility of co-infection of TB and NTM in some of these patients, especially in patients 2 and 3, who were infected with *M. avium* and *M. intracellulare*, which have not been reported to induce a positive IGRA result.

In this study, qPCR was applied to validate the results of mNGS in nine patients, and only six samples were detected to be positive. Several articles have also indicated that the positive rate of mNGS was higher than qPCR in the ability to detect specific pathogens; for example, the Torque teno virus in children with leukemia (Leijonhufvud et al., 2022) and *Orientia tsutsugamushi* (Liu et al., 2021). Besides the positive rate, mNGS also exhibited several other advantages over qPCR in the detection of infectious diseases. First, the discovery power of mNGS was higher than that of qPCR. qPCR can only detect sequences of known pathogens, which were highly suspected by the clinics. In contrast, mNGS is a hypothesis-free approach capable of identifying almost all microbes (DNA and RNA viruses, parasites, fungi, and bacteria) in samples and does not need prior sequence information knowledge, making it especially beneficial for the etiological diagnosis of rare and critical diseases (Ramachandran and Wilson, 2020). Second, mNGS can be characterized as being high throughput. A single mNGS experiment can detect more than one clinical specimen. Using the Illumina NextSeq 550 DX sequencer as an example, which was the mainstream sequencer in the industry of mNGS, a single run can generate more than 400 million reads per run and, thus, can detect as high as 20 samples in parallel (Diao et al., 2022). Meanwhile, a single qPCR experiment can only detect one sample. Therefore, mNGS is suitable for pathogen identification in rare, critical, and difficult-to-detect diseases.

Bronchiectasis is often seen in NTM-PD patients. Previous studies have shown that the incidence of NTM-PD in bronchiectasis patients was significantly higher than in those without bronchiectasis (Aksamit et al., 2017). Furthermore, pre-existing bronchiectasis was deemed as an important risk factor for the occurrence of NTM-PD (Yang et al., 2021). Meanwhile, patients with NTM-PD can also develop bronchiectasis because of repeated infections, which can cause permanent inflammation and scarring of the airways (Chalmers and Sethi, 2017). In our study, we found that only one patient with evidence of bronchiectasis on their chest CT scan had a prior history of bronchiectasis, and for the remaining patients, the CT imaging of bronchiectasis did not ameliorate after treatment (although other patterns of abnormalities showed significant absorption), and this further supported the possible role of NTM infection in causing bronchiectasis directly. Furthermore, pathological findings of bronchiectasis, including bronchial cartilage and smooth muscles layer destruction, airway obstruction by granulomas, and bronchial mucosa ulceration, have been observed in patients diagnosed with NTM-PD(Fujita et al., 2003). In addition, given the generally relative low virulence and slow growth (Ratnatunga et al., 2020), NTM was generally considered to be of low pathogenicity to humans (van Ingen, 2013), the chronic infection was usually accompanied with fibrosis, which could dilate the bronchi with its mechanical traction; this was in accordance with the presence of multifocal bronchiectasis within the focal lesion with little peripheral exudation in our study. So, for patients without prior history of bronchiectasis, NTM infection might be a cause of bronchiectasis.

Our results demonstrated that acute infectious indices, such as WBC count and PCT, were nearly normal, whereas the inflammatory indices including NE percentage, CRP, and ESR were moderately increased in some patients. This, to some degree, provides additional evidence of the relatively low virulence and slow growth of NTM. However, other factors including comorbidities, such as the acute exacerbation of COPD, autoimmune diseases, and use of glucocorticoids and immunosuppressants, can also affect the infectious and inflammatory indices. In addition, lymphocytes, $CD8^+$ T-cell counts, and, especially, $CD4^+$ T-cell counts, were found to be low in patients with NTM-PD, indicating the impaired immunity of these patients. Another noticeable feature was the low BMI in these patients, which was in consistent with previous studies (Song et al., 2021). A lower BMI indicated the poor nutritional status of patients, which was a risk factor for the development of NTM-PD, and was correlated with poor progression (Kim et al., 2017). Thus, clinicians should pay attention to the nutritional status of individuals with predisposing factors for NTM-PD.

There are several limitations to our study. First, because of the retrospective study design, and the fact that our study was performed in a non-referral institution for NTM-PD, only one patient had a positive mycobacterial culture result; thus, a direct comparison of clinical performance between mNGS and culture was not available. Second, several laboratory tests were not taken or missed in the patients' medical record, including standard polymerase chain reaction (PCR) panel analysis for respiratory tract pathogens, and $CD4^+$ and $CD8^+$ T lymphocyte tests. Third, because of the small sample size from a single center, all results were formed through descriptive analysis and the interpretation of our findings was to some degree limited.

Data availability statement

The data presented in the study are deposited in the NCBI Sequence Read Archive database (https://www.ncbi.nlm.nih.gov/sra) with the accession number from SRR18576135 to SRR18576144, and SRR22346179.

Ethics statement

The studies involving human participants were reviewed and approved by the Second Clinical Hospital of Chongqing Medical University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JL designed research. JL and XW collected data. JL and JW analyzed data. JW and HX wrote the paper. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Natural Science Foundation of Chongqing (cstc2019jcyj-msxmX0849) and the Chongqing Medical Scientific Research Project (Joint Project of Chongqing Health Commission and Science and Technology Bureau) (2019QNXM004).

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1083497/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 27 January 2023
DOI 10.3389/fcimb.2023.1106097

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 23 November 2022
ACCEPTED 16 January 2023
PUBLISHED 27 January 2023

CITATION
Zhang B, Chen X, Yao X, Li M, Li Z, Liu B, Liu S, Liu Z, Huo J and Han Y (2023) The diagnostic value of blood metagenomic next-generation sequencing in patients with acute hematogenous osteomyelitis. *Front. Cell. Infect. Microbiol.* 13:1106097. doi: 10.3389/fcimb.2023.1106097

The diagnostic value of blood metagenomic next-generation sequencing in patients with acute hematogenous osteomyelitis

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Aims: This study aims to evaluate the diagnostic value of blood metagenomic next-generation sequencing (mNGS) in detecting pathogens from patients clinically diagnosed as acute hematogenous osteomyelitis (AHO).

Methods: This retrospective study enrolled 66 patients with AHO. The test results of mNGS and bacterial culture on different samples, including blood and puncture fluid samples, from patients with AHO were compared to explore the diagnostic value of blood mNGS. Besides, this study also explored the efficacy of blood mNGS in decision making for antibiotic administration and analyzed the factors associated with the positive result of blood mNGS.

Results: The most common causative pathogens were *Staphylococcus* and *Streptococcus*. The sensitivity of blood mNGS (77.3%) was higher than that of blood culture (42.4%) (P<0.001), while the turnaround time of blood mNGS (2.1 ± 0.4 d) is much less than that of blood culture (6.0 ± 2.1 d) (P<0.001). Besides, the sensitivity of blood mNGS tests (77.3%) was slightly lower than that of puncture fluid mNGS (89.4%). Furthermore, detection comparison at pathogen level unravels that blood mNGS might be suitable for diagnosing AHO caused by common pathogens, while puncture fluid mNGS could be considered as preferred examination in diagnosing AHO caused by uncommon pathogens. Finally, three independent factors associated with the true positive result of blood mNGS in patients with AHO were identified, including Gram-positive pathogens (OR=24.4, 95% CI = 1.4-421.0 for *Staphylococcus*; OR=14.9, 95%CI= 1.6-136.1 for other Gram-positive bacteria), body temperature at sampling time (OR=8.2, 95% CI = 0.6-107.3 for body temperature of >38.5°C; OR=17.2, 95% CI = 2.0-149.1 for patients who were chilling), and no use of antibiotics before sampling (OR=8.9, 95% CI =1.4-59.0).

Conclusion: This is the first report on evaluating and emphasizing the importance of blood mNGS in diagnosing AHO. Blood sample might be an alternative sample for puncture fluid for mNGS, and its extensive application in diagnosing AHO could be expected.

KEYWORDS

diagnostic test, metagenomic next-generation sequencing (mNGS), infection, osteomyelitis (OM), blood

Introduction

Generally, based on the typical clinical manifestation, such as high fever, local tenderness, and elevated erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level, the diagnosis of acute hematogenous osteomyelitis (AHO) is easy to be established (Funk and Copley, 2017). Without rapid etiological diagnosis and proper therapy, AHO may progress to chronic osteomyelitis (Alvares and Mimica, 2020; Wang and Zhang, 2022; Donders et al., 2022) which is difficult to be cured, or may even result in amputation. Therefore, the early diagnosis and treatment of AHO is crucially important for improving the prognoses of patients. However, how to rapidly and accurately identify the infectious pathogens is still a challenging problem that orthopaedic surgeons have to face seriously, since a significant part of AHO are not caused by *Staphylococcus aureus* (Castellazzi et al., 2016).

Bacterial culture is a commonly used method for pathogen detection. The samples used for culture could be blood, puncture fluid, or debridement fluid samples. However, low sensitivity limits its extensive application in accurate diagnosis and treatment. Alex et al. reported that the sensitivity of blood culture in patients with AHO was only 10-50% (Gornitzky et al., 2020). Although the sensitivities of puncture fluid culture or debridement fluid culture were relatively higher, the sampling processes are invasive, and the turnaround time is relatively long. In addition, during this period, the empirical antibiotic treatment might be ineffective, significantly affecting the prognoses of patients.

Therefore, how to rapidly and accurately determine the infectious pathogens is still demanded. Metagenomic next-generation sequencing (mNGS), allowing for high throughput DNA sequencing of pathogen nucleic acid, has been widely used for the detection of microbes and found to be a highly efficient tool for the identification of pathogens. mNGS showed more obvious advantages than culture in detecting pathogens, especially when bacterial load is low, significantly empowering the accurate diagnosis of related diseases (Fang et al., 2022). Some studies had demonstrated that the mNGS of pathogen-rich puncture fluid samples could help in detecting pathogens from patients with AHO (Ramchandar et al., 2021). For example, Zhang et al. found that cryptococcal osteomyelitis could be diagnosed by mNGS when the culture was negative (Zhang et al., 2019). Besides, mNGS showed slightly greater advantage in detecting pathogens from osteoarticular infection samples than conventional culture (88.5% vs 69.2%, $p < 0.01$), especially for the patients having previously received antibiotic treatment (Huang et al., 2020). Given blood sampling is noninvasive and pathogens of AHO are derived from blood, it raises a question that whether blood mNGS can accurately diagnose AHO. However, there are few researches on investigating the performance of blood mNGS in detecting pathogens from patients with AHO.

For infectious diseases in other systems, the advantages of blood mNGS in pathogen detection have already been demonstrated. For instance, Chen et al. reported that bloodstream infection in patients with severe pneumonia could be rapidly diagnosed by blood mNGS (Chen et al., 2021). Besides, Nan et al. also found that blood mNGS was an effective method to identify microorganisms from immunocompromised children with bloodstream infections (BSI) (Nan et al., 2022). Hence, we hypothesized that AHO could be diagnosed by blood mNGS.

To verify our hypothesis, this retrospective study was conducted to evaluate the performance of blood mNGS in detecting pathogens from patients clinically diagnosed as AHO against culture and puncture fluid mNGS. The main research questions are: (1) to obtain the sensitivity of blood mNGS in patients with AHO; (2) to determine whether blood mNGS could correctly identify the infectious pathogens; (3) to dig out which factors may influence the positive rate of blood mNGS.

Material and methods

Patient recruitment

The patients admitted to the Third Hospital of Hebei Medical University from January 1, 2021, to May 31, 2022 and diagnosed as AHO were included in the study. The clinical diagnosis of AHO was based on the clinical practice guideline of the Pediatric Infectious Diseases Society and the Infectious Diseases Society of America (IDSA). Medical history, clinical manifestation, laboratory examination, and imaging diagnosis were summarized. Inclusion criteria of patients were as follows: (1) acute osteomyelitis, with infection duration prior to treatment less than two weeks; (2) patient who undergone blood culture and mNGS; and (3) patients with definite bacteriological diagnosis, which was established *via* both blood sampling, and local puncture sampling or debridement surgery. Exclusion criteria of the patients were as follows: (1) patients with chronic recurrent multifocal osteomyelitis [CRMO] or Bacillus Calmette Guerin osteomyelitis; (2) patients with long term antibiotic medication therapy before osteomyelitis onset; and (3)

lack of definite clinical or bacteriological diagnosis, namely, suspected osteomyelitis or undetermined bacteriological diagnosis.

Sample size and study design

The sample size was estimated prior to the inclusion of these patients. The StatBox (https://www.biostats.cn/statbox/), an online statistical computing system, was used for estimation of sample size. It was assumed that the sensitivities of blood culture and blood mNGS were 0.4 and 0.75, respectively. When the significance level and power of the test were set to 0.05 and 0.8, respectively, the number of needed patients was 31 using Pearson chi-square estimation. To ensure the accuracy of data produced, we finally included 66 patients in our study.

According to the aims of this study, we summarized the performance of culture and mNGS tests using blood and puncture fluid samples. Besides, the study also explored the efficacy of blood mNGS in making decision for antibiotic administration and the factors associated with the positive rate of blood mNGS.

Ethics statement

This study conducted in accordance with the Declaration of Helsinki was approved by the Institutional Review Board of the Third Hospital of Hebei Medical University. Because this was a retrospective study and the information of all patients was deidentified before analysis, this exemption from requiring informed consent was granted by the Ethics Committee of the Third Hospital of Hebei Medical University.

Sample collection

Blood and puncture fluid samples of each patient were collected and further used for pathogen detection by both mNGS and bacterial culture. The acquisition of samples was conducted following a standard operating procedure (SOP). It was briefly described here: The sampling was performed in a treatment room, which was disinfected using ultraviolet light for at least 1 hour before sampling. Median cubital vein was selected as blood sampling sites, which were sterilized at least three times (15 cm around the puncture point) with cotton balls dipped with 0.2% iodine and 75% ethanol. Tourniquet was fastened before the skin puncture and released after the success of puncture. And then the blood sample was saved in a sterilized vacuum blood collection tube without anti-coagulation.

After the suspected diagnosis establishment, the first blood sampling was immediately performed and then at two-hour intervals thereafter. This blood sample acquisition was conducted three times on each patient. The collected samples were divided into two parts. One was used for mNGS detection and the other was used for bacterial culture and antibiotic susceptibility test. Note that, the first blood sampling was conducted before antibiotic treatment in our institute, while the antibiotic treatment was immediately performed after the first sampling.

Puncture fluid samples were collected at the site with the most obvious tenderness by layered sampling method within 72 hours of patients' admission. This was also performed in the same treatment room. The local skin was sterilized at least three times (15 cm around the puncture point) with cotton balls dipped with 0.2% iodine and 75% ethanol, and 1% lidocaine was used for local anaesthesia. Skin was punctured using a pin with a diameter of 2 mm and the fluid was drawn from the soft-tissue and cancellous bone. The samples were also divided into two parts, for mNGS detection and bacterial culture.

Clinical data collection

Clinical data was extracted from medical records, including demographic characteristic (age and sex), clinical information, laboratory test results (blood cell count, CRP, ESR, procalcitonin (PCT)), and detection of infectious pathogens. Body temperature recorded at the first blood sampling was collected for further analysis. Fasting vein blood samples were routinely collected on the first morning of hospitalization for laboratory test, the results of which were recorded in the study. In addition, the administration of antibiotics before admission was summarized for each patient.

mNGS

The process of mNGS was described as follows: (1) DNA extraction: DNA from blood and puncture fluid samples was extracted using the Tianamp micro DNA Kit (DP316, Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. (2) Construction of DNA libraries and sequencing: DNA library construction included fragmenting DNA, repairing end-performing phosphorylation, ligating adaptor, and amplifying polymerase chain reaction (PCR) amplification. The quality of DNA library was evaluated using Qubit (Thermo Fisher Scientific, MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All qualified libraries were sequenced on Nextseq 550 platform (Illumina, San Diego, USA). (3) Bioinformatics analysis: The high-quality sequencing data was obtained after removing low-quality and short (length < 35 bp) reads and following eliminating human host sequences. Such high-quality data was finally blasted against microbial databases, including bacterial, viral, fungal, and parasitic databases.

Diagnostic value of mNGS

In this study, the clinical bacteriological diagnosis determined by clinical surgeons according to the clinical manifestation, laboratory examination, imaging diagnosis, culture and mNGS results of different samples, the treatment effect of antibiotics, and clinical experiences, was set as "gold standard" to evaluate the accuracy of mNGS. The primary index of the study was the sensitivity of blood mNGS. The secondary indexes included misdiagnosis rate and diagnosis time.

As mentioned above, blood sample acquisition was conducted three times on each patient and the three blood samples were all used

for culture, while only the first blood sample collected was used for mNGS test. Both puncture fluid mNGS and culture were performed once. Positive results of mNGS or culture were defined as that the infectious pathogen was correctly determined by mNGS or culture, while misdiagnosis results were defined as that the results of mNGS or culture were inconsistent with clinical bacteriological diagnosis ('gold standard' above). For blood mNGS, puncture fluid mNGS, and puncture fluid culture, sensitivity and misdiagnosis rate are the numbers of positive results and misdiagnosis results divided by the number of enrolled patients, respectively. For blood culture, the numbers of positive results and misdiagnosis results divided by the total number of blood culture performed on the enrolled patients are sensitivity and misdiagnosis rate, respectively. Diagnosis time was identified as the time period from sampling to result report. Besides, the factors which may influence the positive result of blood mNGS were analyzed.

Statistical analysis

Continuous variables were expressed as the mean ± standard deviation [SD], while the categorical variables were presented as count (proportion) when appropriate. Sensitivity, total sensitivity, misdiagnosis rate, and diagnosis time were compared between mNGS and bacterial culture on different samples by applying Pearson's chi-squared test, McNemar test and paired *t*-test as appropriate. Logistic regression was used for analyzing the factors which may influence the positive result of blood mNGS. A *P* value less than 0.05 was considered to be significant. The above statistical analyses were conducted by using SPSS 19.0 statistical software for Windows (IBM, Armonk, NY) and Excel 2022 for Windows (Microsoft Corporation, Seattle, WA).

Data availability

Sequencing data were deposited to the National Genomics Data Center under accession number PRJCA013362. The authors declare that the main data supporting the findings are available within this article. The other data generated and analyzed for this study are available from the corresponding author upon reasonable request.

Result

Demographic and clinical features of patients

A total of 66 patients diagnosed with AHO were enrolled in the current study. The median age of the enrolled patients was 10 years old, and 37 (56.1%) patients were male (Table 1). The most commonly infected bone was distal femur (*n* =29, 43.9%), followed by proximal tibia (*n* =26, 39.4%). The mean follow-up period was 18.7 ± 3.8 months. The infection parameters of patients with AHO were assessed (Table 2). In addition to fever, some AHO patients had other symptoms, including chills, soft tissue erythema, swollen and draining sinus tracts, *etc.* The blood testing of patients with AHO showed the classic manifestation of bacterial infection. Blood cell counts such as leukocytes ((10.8 ± 1.9) $\times10^9$/L) and neutrophils ((8.2 ± 2.0) $\times10^9$/L, 75.0 ± 6.5%) were at relatively high level. Moreover, the CRP was at a high level (61.0 ± 35.3 mg/L), as was the ESR (63.0 ± 23.2 mm/h). The mean time period from the onset of symptom to the first blood sampling was 77.6 ± 31.0 hours. More than half of the patients (*n* = 37) developed fever or chilling. The temperatures of 17 (25.8%) and 20 (30.3%) patients at the time of sampling were < 38.5°C and ≥38.5°C, respectively, while 29 patients were chilling (43.9%). In addition, 13 patients (19.7%) received antibiotic treatment before sampling (Table 2).

TABLE 1 Demographic characteristics and clinical information of the patients with AHO.

Characteristics	Number of cases
Age (years)	
1-3 years	7
4-6 years	12
7-12 years	28
13-16 years	13
>16 years	6
Sex	
Male	37
Female	29
Weight (kg)	37.6 ± 17.3
Body mass index	19.6 ± 8.8
Affected bone	
Distal femur	29
Proximal tibia	26
Other metaphysis of long tube bone	5
Shaft of long tube bone	4
Other	2
Follow-up time (months)	18.7 ± 3.8

Pathogen profile

Combined with the clinical manifestation, laboratory examination, imaging diagnosis, culture, mNGS results, and clinical experience, we defined causative pathogens of AHO (Table 2). It was found that all of the causative pathogens were bacteria, of which Gram-positive bacteria accounted for 63.6%. The common causative pathogens were *Staphylococcus* (*n* = 19) and *Streptococcus* (*n* = 17), followed by *Klebsiella* (*n* = 7), *Pseudomonas aeruginosa* (*n* = 7), *Enterococcus* (*n* = 6), *Acinetobacter* (*n* = 3), *Haemophilus influenzae* (*n* = 3), and *Escherichia* (*n* = 2). Besides, *Brucella* (*n* = 1) and *Mycobacterium tuberculosis* (*n* = 1) were also proven to cause AHO.

10.3389/fcimb.2023.1106097

TABLE 2 Infectious parameters in patients with AHO.

Parameters	
Time period from the onset of symptom to first blood sampling (hours)	77.6 ± 31.0
Body temperature at sampling	
<38.5°C	17
≥38.5°C	20
Chilling	29
Antibiotics administration before admission (*n*)	
No	53
Yes	13
Average weighted temperature (degree of centigrade)	38.5
Blood cell count	
Leukocytes	10.8 ± 1.9
Hemoglobin (g/L)	136.9 ± 12.2
Neutrophils	8.2 ± 2.0
Neutrophils, %	75.0 ± 6.5
Lymphocytes	1.9 ± 0.4
Monocytes	0.4 ± 0.2
Platelets	298.8 ± 29.0
C-reactive protein (mg/L)	61.0 ± 35.3
Erythrocyte sedimentation rate (mm/h)	63.0 ± 23.2
Procalcitonin (ng/mL)	0.2 ± 0.1
Infectious bacteria (*n*)	
Acinetobacter	3
Brucella	1
Mycobacterium tuberculosis	1
Enterococcus	6
Enterobacteriaceae	2
Haemophilus influenzae	3
Klebsiella	7
Pseudomonas aeruginosa	7
Staphylococcus	19
Streptococcus	17

Comparison between blood mNGS and culture

Compared with conventional blood culture, blood mNGS can accurately detect more pathogens (Table 3, Figure S1). The sensitivity of blood mNGS was 77.3%, which was higher than that of blood culture (42.4%) (P <0.001). There was no significant difference in the misdiagnosis rate between blood mNGS and blood culture (6.1% *vs* 3.5%, P=0.474). However, the turnaround time of mNGS is much less than that of blood culture (2.1 ± 0.4 *vs* 6.0 ± 2.1 days, P<0.001).

Advantages of blood mNGS and puncture fluid mNGS

To evaluate the performance of blood mNGS in diagnosing AHO, puncture fluid sample was simultaneously collected for mNGS test (Figure S2). It was found that the sensitivity of blood mNGS (77.3%) was slightly lower than that of puncture fluid mNGS (89.4%) and puncture fluid culture (87.9%) (Table 3). Besides, as focal sample, misdiagnosis rates of puncture fluid mNGS (4.65%) and puncture fluid culture (1.5%) were slightly lower than that of blood mNGS (6.1%). High performance and low misdiagnosis rate indicate that blood sample might be an alternative sample for puncture fluid for mNGS in diagnosing AHO.

Pathogen detection by different methods

We investigated the detection performance of pathogens by blood mNGS, blood culture, puncture fluid mNGS, and puncture fluid culture (Figure 1). Blood mNGS and puncture fluid mNGS can simultaneously detect pathogens from most of the enrolled patients (n =45), while there was only 1 patient with both blood and puncture fluid mNGS negative. For the patients (n =14) with blood mNGS negative (-) and puncture fluid mNGS positive (+), *Staphylococcus* (n =3) and *Klebsiella* (n =3), *Pseudomonas aeruginosa* (n =2), *Acinetobacter* (n =2), *Haemophilus influenzae* (n =2) were detected. While for the patients with blood mNGS positive (+) and puncture fluid mNGS negative (-), *Streptococcus* (n =2) and *Staphylococcus* (n =3) were identified. The above results indicate that blood mNGS might be much more suitable for detecting common pathogens of AHO, rather than uncommon pathogens.

We further summarized positive coincidence rates of different methods at the pathogen level. For *Staphylococcus*, while the positive coincidence rate of blood culture (47.4%) was less than 50%, 84.2% of results revealed by blood mNGS were consistent with final clinical diagnoses. Besides, most of *Staphylococcus* can be detected by puncture fluid culture (94.7%), and the positive coincidence rate of puncture fluid mNGS was the same as that of blood mNGS. A same trend was found in the detection of *Streptococcus*. Interestingly, positive coincidence rate of blood mNGS (94.1%) for *Streptococcus* was higher than that of puncture fluid mNGS (88.2%).

For *Pseudomonas aeruginosa*, positive coincidence rate of puncture fluid mNGS can reach up to 100%, higher than that of blood mNGS (71.4%). Puncture fluid culture can successfully detect *Pseudomonas aeruginosa* from 85.7% of patients, while only 28.6% of patients can be accurately diagnosed by blood culture. Same trends were also found in the detection of *Klebsiella*, *Enterococcus*, and other uncommon pathogens of AHO. The above results provide explanation for our finding that blood mNGS was suitable for detecting common pathogens of AHO, and further unravel that blood and puncture fluid sample might be considered as preferred sample for mNGS in detecting AHO caused by common and uncommon pathogens, respectively.

TABLE 3 Comparison of mNGS diagnostic value between blood and puncture samples.

Diagnostic parameters	Blood sample				Puncture fluid sample			
	mNGS	Bacterial culture	Test statistics	*P*	mNGS	Bacterial culture	Test statistics	*P*
Sensitivity (First sampling)	77.3%	42.4%	16.7	<0.001	89.4%	87.9%	0.08	0.784
Total Sensitivity (First three samplings)	–	63.6%	–	–	–	–	–	–
Misdiagnosis (%)	6.1%	3.5%	–	0.474	4.7%	1.5%	–	0.619
Diagnosis time (days)	2.1 ± 0.4	6.0 ± 2.1	-10.8	<0.001				

Factors associated with mNGS sensitivity

Three independent factors identified were associated with the true positive result of blood mNGS in patients with AHO, including detected pathogens, body temperature at sampling time, and antibiotic administration before sampling (Table 4). Compared with Gram-negative bacteria, the estimated odds ratio for *Staphylococcus* was 24.4 (95% CI = 1.4-421.0, *P*=0.028) in this cohort of patients with AHO, and it was 14.9 (95% CI = 1.6-136.1, *P*=0.017) for other gram-positive bacteria. Another independent factor was body temperature

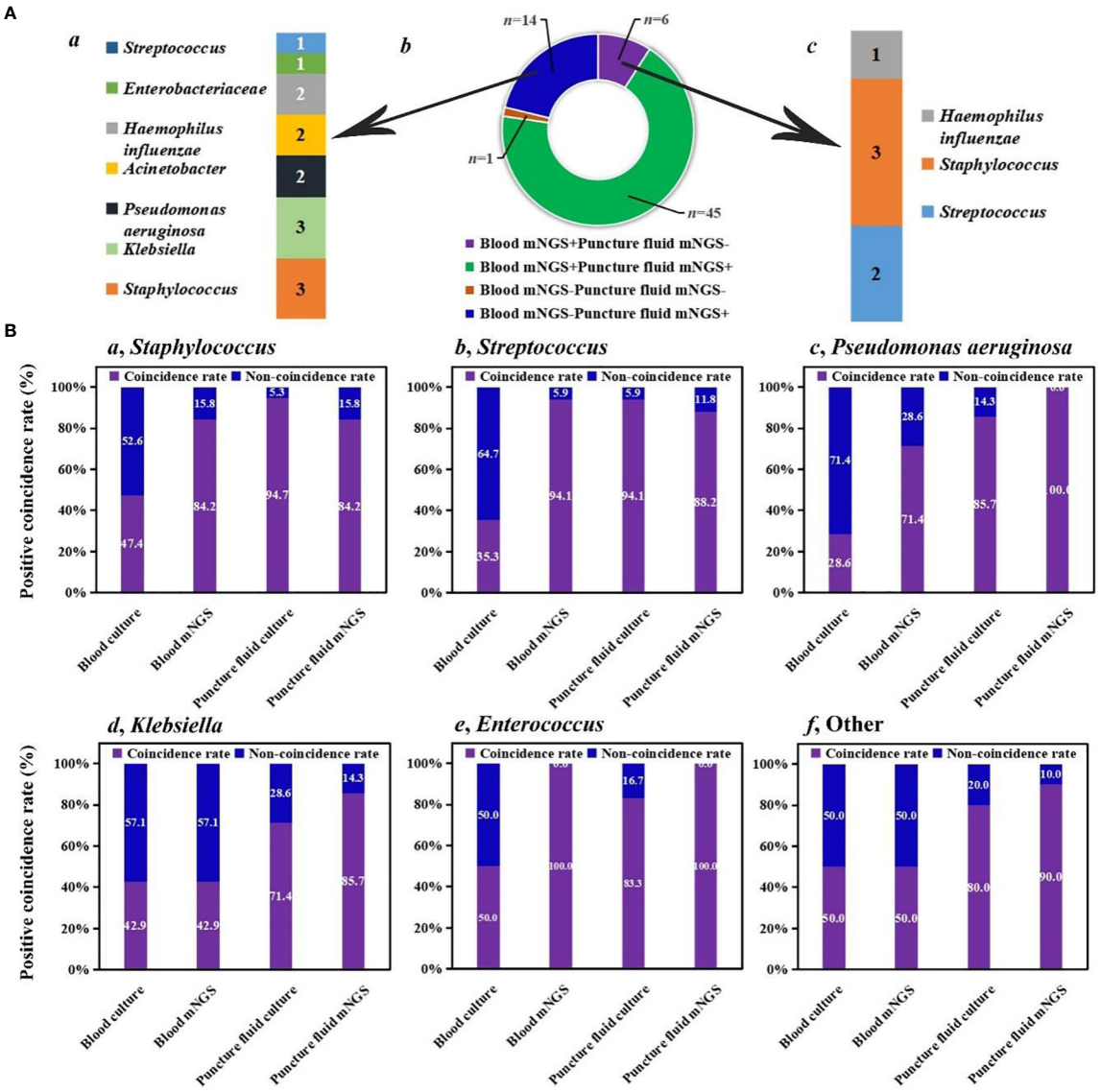

FIGURE 1
Comparison among different methods. **(A)** Detection performance of blood and puncture fluid mNGS. **(B)** Coincidence rates of different methods against final clinical diagnoses.

TABLE 4 Factors associated with the positive results of mNGS in patients with AHO.

Factors	Odds ratio	95% confidence interval for odds ratio	*P*
Infected bacteria			
Staphylococcus	24.4	1.4-421.0	0.028
Other Gram-positive bacteria	14.9	1.6-136.1	0.017
Other Gram-negative bacteria	Ref.		
Body temperature at sampling			
Chilling	17.2	2.0-149.1	0.010
≥38.5°C	8.2	0.6-107.3	0.111
<38.5°C	Ref.		
Antibiotics administration before sampling			
Yes	Ref.		
No	8.9	1.4-59.0	0.023

at sampling. Compared with body temperature of <38.5°C, the estimated odds ratio for body temperature of ≥38.5°C and chilling were 8.2 (95% CI = 0.6-107.3, *P*=0.111) and 17.2 (95% CI = 2.0-149.1, *P*=0.010), respectively, indicating that higher body temperature or chilling may cause a rise in mNGS sensitivity. Besides, compared with antibiotic administration before sampling, the estimated odds ratio for the patients without antibiotic treatment was 8.9 (95% CI = 1.4-59.0, *P*=0.023), suggesting that antibiotic treatment before sampling may cause a decrease in the positive rate of mNGS.

Discussion

To the best of our knowledge, this is the first report on evaluating the performance of blood mNGS in patients with AHO. The common causative pathogens of AHO were *Staphylococcus* and *Streptococcus*. Taking final clinical diagnoses as gold standard, we found that the sensitivity of blood mNGS (77.3%) was slightly lower than that of puncture fluid mNGS (89.4%). Furthermore, detection comparison at pathogen level unravels that blood mNGS might be suitable for diagnosing AHO caused by common pathogens, while puncture fluid mNGS could be considered as preferred examination in diagnosing AHO caused by uncommon pathogens.

We proved that blood mNGS could be used for diagnosing AHO, challenging the long-held opinion that puncture fluid sample is essential for mNGS test in detecting pathogens of AHO. For conventional methods, the most suitable sample for infection diagnosis is focal sample, while low or zero pathogen loads in blood limit its extensively application in accurate infection diagnosis. Haessler et al. found that *Streptococcus pneumoniae* (33%) and *S. aureus* (22%) causing severe pulmonary infection can be successfully isolated from blood samples, indicating that pathogens in focal site spread to bloodstream and blood sample might be considered as an alternative sample for diagnosing severe pulmonary infection (Haessler et al., 2020). While for hematogenous infection, the clinical problem is whether the microbes detected from blood samples are causative pathogens, rather than whether the microbes could be detected. Rakow et al. found that more than 61% of pathogen (43/70) causing hematogenous periprosthetic joint infection can be successfully detected from blood samples (Renz et al., 2022). For AHO, we found that the coincidence rate (sensitivity) of blood mNGS tests (77.3%) against final clinical diagnosis was slightly lower than that of puncture fluid mNGS (89.4%). Interestingly, positive coincidence rate of blood mNGS (94.1%) for *Streptococcus* was higher than that of puncture fluid mNGS (88.2%), which might be because 1) the *Streptococcus* was not collected from the focal site during the puncture fluid sampling or 2) *Streptococcus* load in some puncture fluid sample was too low to be detected by mNGS. According to our results, we propose that blood sample might be an alternative sample of puncture fluid for mNGS in diagnosing AHO.

In addition, the diagnosis time of blood mNGS (2.1 ± 0.4 d) was significantly shorter than that of blood culture (6.0 ± 2.1 d) in this study. Grumaz et al. reported that mNGS detection only took 30 h (turnaround time) to accomplish the whole process from blood sample preparation to pathogens identification report (Grumaz et al., 2019). For culture, although the growth time of Gram-negative bacteria and Gram-positive bacteria is 11 h (10-13 h) and 12 h (12-18 h), respectively, the turnaround time of bacterial culture is more than 7 d, which is much longer than growth time (Mendoza et al., 2015). Therefore, mNGS could be considered to be a promising tool for guiding the timely and accurate use of antibiotics.

Finally, this study explored the influence factors associated with positive result of mNGS, which may be crucial for reasonable interpretation of results and clinical application. An early study suggested that a blood culture was more likely to show positive results if it was taken prior to a temperature spike, which was further confirmed by the study of Kee et al. that bacteremia preceded a fever (Kee et al., 2016). We also found that higher body temperature or chilling may cause a rise in mNGS sensitivity. Moreover, previous study reported that the mNGS performance is less likely to be affected by prior use of antibiotics, which was contrary to the results of our study (Miao et al., 2018). In the treatment of osteomyelitis, β-lactams were listed as the first choice of antibiotic usage (Nade, 1977; Autore et al., 2020). It was found that β-lactams can kill pathogen *via* destroying their cell walls, decreasing the abundance of pathogen DNA in bloodstream and resulting in negative result of subsequent mNGS detection. Understanding factors associated with the positive result of blood NGS could potentially promote the clinical diagnostic value of blood mNGS.

The limitations of this study are as follows. (1) The current sample size is relatively small and further studies should enroll more patients to generate more accurate findings. (2) The study was conducted by retrospective analysis, which may limit the comprehensive analysis or result in information bias. (3) The analysis of mNGS cost was not performed, which may limit its clinical application.

Conclusions

The study demonstrated that blood mNGS did exhibit good performance in identifying the pathogens from patients with AHO. Taking final clinical diagnoses as gold standard, the sensitivity of blood mNGS (77.3%) was slightly lower than that of puncture fluid

mNGS (89.4%). Furthermore, blood mNGS and puncture mNGS might be suitable for diagnosing AHO caused by common pathogens and uncommon pathogens, respectively. Besides, antibiotic treatment before sampling may cause a decrease in the positive rate of mNGS.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Because this was a retrospective study and the information of all patients was identified before analysis, this exemption from requiring informed consent was granted by the Ethics Committee of the Third Hospital of Hebei Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

YH designed the paper. BZ and XC drafted the manuscript. XY, ML, and ZhL carried out the clinical care and management of the patients. BL and BZ performed the mNGS tests and analyzed the data. ZeL and JH revised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1106097/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 10 February 2023
DOI 10.3389/fcimb.2023.1117987

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 07 December 2022
ACCEPTED 30 January 2023
PUBLISHED 10 February 2023

CITATION
Qin C, Zhang S, Zhao Y, Ding X, Yang F and Zhao Y (2023) Diagnostic value of metagenomic next-generation sequencing in sepsis and bloodstream infection. *Front. Cell. Infect. Microbiol.* 13:1117987. doi: 10.3389/fcimb.2023.1117987

Diagnostic value of metagenomic next-generation sequencing in sepsis and bloodstream infection

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Objective: To evaluate the diagnostic value of metagenomic next-generation sequencing (mNGS) in sepsis and bloodstream infection (BSI).

Methods: A retrospective analysis of patients diagnosed with sepsis and BSI at the First Affiliated Hospital of Zhengzhou University from January 2020 to February 2022 was conducted. All the patients underwent blood culture and were divided into mNGS group and non-mNGS group according to whether mNGS was performed or not. The mNGS group was further divided into early group (< 1 day), intermediate group (1–3 days), and late group (> 3 days) according to the time of mNGS inspection.

Results: In 194 patients with sepsis and BSI, the positive rate of mNGS for identifying pathogens was significantly higher than that of blood culture (77.7% vs. 47.9%), and the detection period was shorter (1.41 ± 1.01 days vs. 4.82 ± 0.73 days); the difference was statistically significant ($p < 0.05$). The 28-day mortality rate of the mNGS group ($n = 112$) was significantly lower than that of the non-mNGS group ($n = 82$) (47.32% vs. 62.20%, $p = 0.043$). The total hospitalization time for the mNGS group was longer than that for the non-mNGS group (18 (9, 33) days vs. 13 (6, 23) days, $p = 0.005$). There was no significant difference in the ICU hospitalization time, mechanical ventilation time, vasoactive drug use time, and 90-day mortality between the two groups ($p > 0.05$). Sub-group analysis of patients in the mNGS group showed that the total hospitalization time and the ICU hospitalization time in the late group were longer than those in the early group (30 (18, 43) days vs. 10 (6, 26) days, 17 (6, 31) days vs. 6 (2, 10) days), and the ICU hospitalization time in the intermediate group was longer than that in the early group (6 (3, 15) days vs. 6 (2, 10) days); the differences were statistically significant ($p < 0.05$). The 28-day mortality rate of the early group was higher than that of the late group (70.21% vs. 30.00%), and the difference was statistically significant ($p = 0.001$).

Conclusions: mNGS has the advantages of a short detection period and a high positive rate in the diagnosis of pathogens causing BSI and, eventually, sepsis. Routine blood culture combined with mNGS can significantly reduce the mortality of septic patients with BSI. Early detection using mNGS can shorten the total hospitalization time and the ICU hospitalization time of patients with sepsis and BSI.

KEYWORDS

bloodstream infection, sepsis, blood culture, MNGs, diagnosis, Intensive Care Unit

1 Introduction

Bloodstream infection (BSI) refers to positive blood culture in patients with signs and symptoms of systemic infection, which can be classified as primary, that is, not secondary to an identified infection, or secondary, that is, following a localized infectious disease. As a common life-threatening disease in the intensive care unit (ICU), severe cases of BSI can lead to sepsis and septic shock, with high mortality rates (Massart et al., 2021; Guzek et al., 2022). Early and effective antibiotic course is the key to treating BSI. If the pathogen cannot be identified early in the disease, effective antibiotics will be administered late, which often leads to poor clinical outcomes (Lindberg et al., 2020). In severe sepsis and septic shock, the survival rate of patients decreases by an average of 7.6% every hour of delayed use of effective antibiotics. If proper antibiotic treatment is not given within 1 hours, the survival rate of sepsis can decrease (Andersson et al., 2019; Sanguanwit et al., 2022). The current guidelines recommend that antibiotic treatment be started as early as 1 hour after diagnosing sepsis or septic shock (Evans et al., 2021). However, in actual clinical work, about 46% of empirical antibiotic treatment is proven inappropriate, leading to an increase in mortality and antibiotic resistance risk, and toxic reactions (Campion and Scully, 2018). Accurate identification of BSI pathogens is of great value for early guidance of antibiotic treatment, better antibiotic management, and improved clinical outcomes.

At present, blood culture is still the gold standard for BSI diagnosis. Expert consensus on bloodstream infection in critically ill patients recommends that at least two sets of aerobic and anaerobic cultures be sampled after skin disinfection following strict aseptic guidelines (Timsit et al., 2020). However, prolonged blood culture tests, low microbial inoculum, or antibiotics before blood draw will reduce the positive rate of blood culture (Pilecky et al., 2019; Rand et al., 2019; Yu et al., 2020). In recent years, metagenomic next-generation sequencing (mNGS) has become a new method for detecting pathogenic microorganisms. It can analyze whole microbial colonies in clinical samples without needing prolonged culture (Miao et al., 2018; Blauwkamp et al., 2019). It has been reported that mNGS can help identify pathogenic microorganisms of suspected BSI patients with high sensitivity and specificity (Grumaz et al., 2019). However, few studies on the clinical application of mNGS in BSI have been carried out, most of which are case reports or small sample studies (Sun et al., 2022). Therefore, it is urgent to evaluate the importance of the mNGS technique in the diagnosis of sepsis and BSI.

2 Materials and methods

2.1 Study subjects

This study is a retrospective analysis of septic patients with BSI from the First Affiliated Hospital of Zhengzhou University. No blood tests or drug interventions were carried out. Electronic medical records of patients were reviewed to collect clinical data and laboratory results. This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University, with the ethics number 2022-KY-0225-002.

2.2 Research methods

2.2.1 Inclusion criteria

Septic patients with bloodstream infection admitted to the First Affiliated Hospital of Zhengzhou University, China, from January 2020 to February 2022, were screened for this study. According to the sepsis 3.0 standard (Singer et al., 2016), sepsis can be diagnosed if the following two conditions are met: Definite or suspected infection exists; sequential organ failure assessment (SOFA) score increased by ≥2 points compared with baseline. Organ dysfunction was assessed by the SOFA scoring system, and disease severity was assessed by the acute physiology and chronic health assessment II (APACHE II) scoring system. Referring to the 2016 US CDC clinical diagnostic criteria for bloodstream infection, BSI can be defined as: Fever ($T \geq 38.0°C$), chills, or accompanied by hemodynamic instability that cannot be explained by infection in a specific body part (CDC, 2016). All patients had their blood taken for culture after admission.

2.2.2 Exclusion criteria

No blood culture was performed after admission; The hospitalization time was less than 24 hours; Age < 18 years old; At the terminal stage of malignant diseases such as decompensated cirrhosis and malignant tumor, the expected survival time was no more than 3 months; Missed follow-up.

2.3 Analysis of information collected

The clinical data collection of the selected subjects included: age, gender, ICU hospitalization time, total hospitalization time, mechanical ventilation time and vasoactive drug use time, past medical history, laboratory results within 24 hours after admission (white blood cell, red blood cell, platelet, hemoglobin, serum creatinine, urea nitrogen, alanine aminotransferase, glutamic oxaloacetic transaminase, total bilirubin, albumin, plasma prothrombin time, activated partial thromboplastin time, and international standardized ratio), blood culture results (pathogen type, submission time, and detection time), mNGS results (pathogen type, sequence number, relative abundance, submission time, and detection time), serum infection markers (erythrocyte sedimentation rate, C-reactive protein, interleukin-6, and procalcitonin), ICU special treatment data (based on antibiotic adjustment (blood culture, mNGS or clinical)), adjustment contents, adjustment time, APACHE II and SOFA scores within 48 hours of admission, and APACHE II and SOFA scores 7 days after antibiotic adjustment.

2.4 Follow-up and grouping

Telephone follow-up was conducted to document patient outcome indicators. The primary outcome was determined by 28-day mortality. The secondary outcome was to determine the ICU hospitalization time, total hospitalization time, mechanical ventilation time, vasoactive drug use time, and 90-day mortality. The patients were divided into mNGS group and non-mNGS group

according to whether they received mNGS or not, respectively. The patients in the mNGS group were further divided into early group (< 1 day), intermediate group (1–3 days), and late group (> 3 days) according to the application time of mNGS.

2.5 Statistical analysis

SPSS 22.0 software was used for data analysis. The Kolmogorov-Smirnov test was used to verify the normality of the measurement data. The data in accordance with the normal distribution were described by the mean and standard deviation (SD). The comparison between groups adopted the T-test or corrected T-test according to the homogeneity of variance. The median (quartile) [M (QL, QU)] was used to describe the distribution characteristics of the non-normal distribution, and the Mann-Whitney U nonparametric test was used for comparison between groups. The Chi-square test was used to compare variables. $p < 0.05$ was considered statistically significant.

3 Results

3.1 Patient baseline characteristics

A total of 216 discharged patients diagnosed with sepsis and bloodstream infection were screened for this study, of which 12 patients did not meet the diagnostic criteria of bloodstream infection, 5 patients did not meet the diagnostic criteria of sepsis, and 4 patients had no blood culture, 1patient was not age-compatible and 4 patients were lost during follow-up(included in the above), resulting in 194 patients with sepsis and BSI remaining in this study, including 128 males (66.0%) and 66 females (34.0%). 112 cases were in the mNGS group (blood culture and mNGS were performed), and 82 cases were in the non-mNGS group (blood culture only). The positive rate of mNGS was 77.7% (87/112), and that of blood culture was 47.9% (93/194). Figure 1 illustrates the inclusion process.

The mean age of the patients was 55.61 ± 15.87 years, and SOFA and APACHE II scores were 8.37 ± 4.67 and 19.27 ± 8.60, respectively. 109 patients died within 28 days, and the 28-day mortality rate was 56.2%; 135 patients (69.6%) received mechanical ventilation, 128 patients (70.0%) received vasoactive drugs, and 80 patients (41.2%) received antibiotics before blood culture.

The average period of mNGS detection was 1.41 ± 1.01 days, and the average time of traditional blood culture detection was 4.82 ± 0.73 days. The time of mNGS detection was significantly shorter than that of traditional blood culture detection ($p < 0.05$).

3.2 Detection of pathogens in blood culture

Among the 194 patients, 93 (47.9%) had a positive blood culture, including 65 cases of Gram-negative bacteria, 22 cases of Gram-positive bacteria, and 6 cases of fungi. Among the Gram-negative bacteria, 26 cases were *Klebsiella pneumoniae* (40.0%), 15 cases were *Escherichia coli* (23.1%), 9 cases were *Acinetobacter baumannii* (13.8%), and 3 cases were *Pseudomonas aeruginosa* (4.6%). Gram-positive bacteria strains were mainly *Staphylococcus aureus* (4 cases, 18.2%), *Staphylococcus epidermidis* (3 cases, 13.6%), and *Staphylococcus haemolyticus* (3 cases, 13.6%). The most common fungi detected were *Candida albicans* (2 cases, 33.3%) and *Candida tropicalis* (2 cases, 33.3%). See Figure 2 for details.

3.3 Pathogens detected by mNGS

We determined pathogens according to the sequence number and relative abundance of detected pathogens in combination with clinical practices. If several pathogens are detected in a blood sample by mNGS, a maximum number of four pathogens with high sequence numbers and relative abundance are clinically suspected. mNGS detected 180 types of suspected pathogens, and the most commonly detected Gram-negative bacteria were *Klebsiella pneumoniae* ($n = 21$), *Acinetobacter baumannii* ($n = 10$), *Escherichia coli* ($n = 6$), and *Pseudomonas aeruginosa* ($n = 3$). The most commonly detected Gram-positive bacteria were *Staphylococcus aureus* ($n = 4$), and *Enterococcus faecalis* ($n = 3$). The most frequently detected fungi were *Pneumocystis Yersinia* ($n = 4$) and *Rhizopus macrocarpa* ($n = 5$). The most commonly detected viruses were human herpesvirus type 5 ($n = 14$), cytomegalovirus ($n = 12$), and Epstein-Barr virus ($n = 11$). See Figure 3 for details.

3.4 Baseline data

The difference in gender, age, APACHE II and SOFA scores 48h after admission between the mNGS group and the non-mNGS group was not significant ($p > 0.05$). In the non-mNGS group, the proportion of stroke patients was significantly higher, and hemoglobin was significantly lower compared to the mNGS group ($p < 0.05$). See Table S1 for details.

3.5 Comparison of primary and secondary outcomes between mNGS group and non-mNGS group

The 28-day mortality rate of the mNGS group was significantly lower than that of the non-mNGS group ($p = 0.043$). The total hospitalization time of the mNGS group was significantly longer than that of the non-mNGS group ($p = 0.005$). There was no significant difference in the ICU hospitalization time, mechanical ventilation time, vasoactive drug use time, and 90-day mortality between the two groups ($p > 0.05$). See Table 1 for details.

3.6 Consistency analysis of blood culture and mNGS pathogen detection

When the pathogen detected by mNGS is the same as that detected by blood culture, the detection results are considered consistent. If mNGS detects more pathogens than the blood culture method does, and at least one of them is consistent with blood culture, the test results are also considered consistent. When no pathogen is

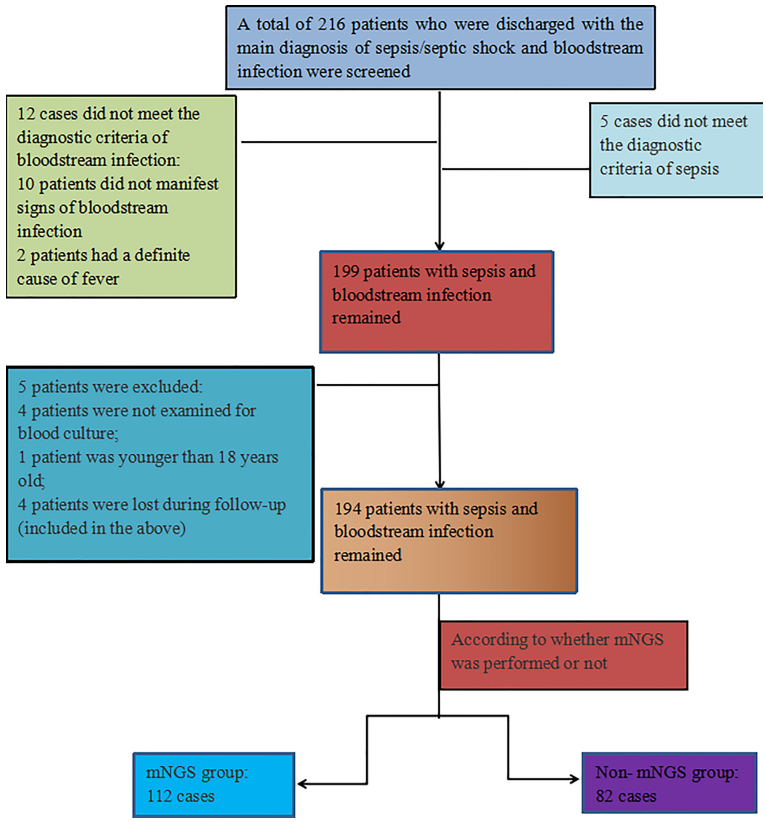

FIGURE 1
Inclusion process chart.

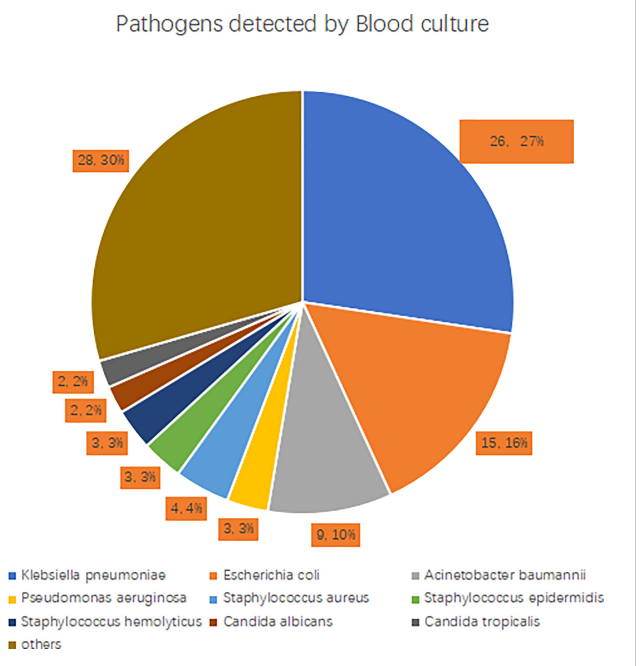

FIGURE 2
Pathogens detected by blood culture.

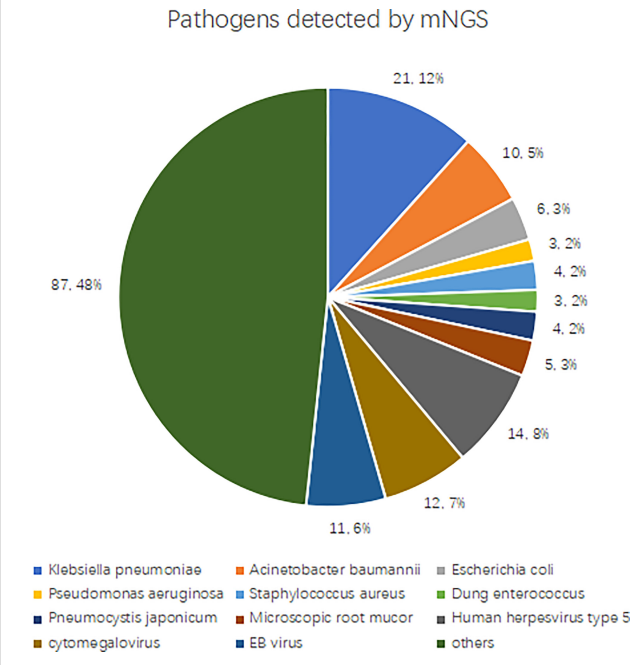

FIGURE 3
Pathogens detected by mNGS.

detected by either method, the test results are considered to be negatively consistent. When the pathogens identified by the two methods are completely different or when one method is positive while the other is negative, the test results are considered inconsistent. 27 cases (24.1%) of pathogens detected in mNGS group were consistent with blood culture results, of which 14 cases (12.5%) were positive and 13 cases (11.6%) were negative. 85 cases (75.9%) were inconsistent, of which 66 cases (77.7%) were negative by the blood culture method, 12 cases (14.1%) were negative by the mNGS method, and 7 cases (8.2%) were inconsistent with the pathogens detected by the two methods. See Figures 4, 5 for details.

3.7 The application of mNGS to guide clinical treatment

The adjustment times of antibiotics in the mNGS group were 2.13 ± 1.15, and those in the non-mNGS group were 1.73 ± 1.40. There was no significant difference between the two groups ($p > 0.05$). 27 patients (13.9%) had their first antibiotic treatment adjusted according to mNGS test results, 15 patients (7.7%) had their first antibiotic treatment adjusted according to blood culture results, and the rest of the patients had their first antibiotic treatment adjusted according to clinical manifestations and overall condition. After the antibiotic regimen was adjusted for the first time, we observed and followed up on the APACHE II and SOFA scores for the next 7 days and found that the difference in APACHE II and SOFA scores between the two groups after 7 days of treatment was not significant ($p > 0.05$). See Table 2 for details.

3.8 Sub-group analysis: The effect of early, intermediate, and late mNGS on patients

According to the mNGS detection time after admission, patients in the mNGS group were divided into the early group (within one day after enrollment), the intermediate group (1–3 days after enrollment), and the late group (3 days after enrollment). There was no significant difference in baseline data between the three groups ($p > 0.05$; see Table S2 for details). However, the 28-day mortality, the ICU hospitalization time, and the total hospitalization time were significantly different among the three groups ($p < 0.05$; see Table 3 for details). There was no significant difference in mechanical ventilation time, vasoactive drug use time, or 90-day mortality among the three groups ($p > 0.05$; see Table 3 for details).

Pairwise comparison was conducted for the outcome indices with statistical differences among the three groups. The results showed no statistical difference in the total hospitalization time between the early group and the intermediate group ($p = 0.418$). The total hospitalization time in the early group was shorter than that in the late group, and the difference was statistically significant ($p = 0.001$). The ICU hospitalization time in the early group was shorter than in the intermediate group, and the difference was statistically significant ($p = 0.005$). The ICU hospitalization time in the early group was significantly shorter compared to the late group ($p < 0.001$). The difference in the 28-day mortality rate between the early and intermediate groups was not statistically significant ($p = 0.025$), but the 28-day mortality rate of the early group was significantly higher than that of the late group ($p = 0.001$).

TABLE 1 Comparison of primary and secondary outcomes between mNGS group and non-mNGS group.

	mNGS group (n = 112)	Non-mNGS group (n = 82)	p
28-day mortality	53 (47.32%)	51 (62.20%)	0.043
Total hospitalization time (d)	18 (9, 33)	13 (6, 23)	0.005
ICU hospitalization time (d)	7 (3, 15.75)	6.5 (3, 15.50)	0.355
Mechanical ventilation time (h)	29.5 (0, 193.50)	46 (0, 144)	0.709
Vasoactive drug use time (h)	27 (0, 142.5)	36 (0, 120)	0.815
90-day mortality	64 (57.14%)	57 (69.51%)	0.099

4 Discussion

The application of mNGS in patients with sepsis and BSI, especially to investigate whether blood culture combined with mNGS can reduce the mortality rate and the clinical implications of early and late mNGS, has rarely been reported. Our study has shown that mNGS has the advantages of a short detection time and a high positive rate in early pathogen diagnosis of BSI and sepsis. Routine blood culture combined with mNGS can reduce the 28-day mortality rate of patients with sepsis and BSI. Early mNGS can shorten the total hospital stay and the ICU hospitalization period.

BSI is a common infectious disease that can eventually lead to severe sepsis and septic shock. It is more likely to occur in patients who have been in ICU for a long time, have had invasive procedures, and are immunosuppressed (Massart et al., 2021). Common pathogens include bacteria, fungi, viruses, and atypical pathogens. For BSIs, broad-spectrum antibiotics or empiric therapy are initially administered before pathogen identification and then adjusted to targeted therapy based on microbiological analysis. Early identification of the pathogen in severe infections resulting in sepsis and septic shock can guide precise treatment (Zhou et al., 2019). Ineffective or inappropriate antibiotic therapy can lead to the generation of multi-drug resistant bacteria, resulting in longer hospital stay, ICU stay, and higher mortality rate (Kumar et al., 2006; Andersson et al., 2019; Raad et al., 2021; Ababneh et al., 2022; Kanj et al., 2022).

Traditional blood culture remains the gold standard for the diagnosis of BSI. However, the positive rate of blood culture is low. It is greatly affected by prior antibiotic use and takes a long time to incubate (Greninger and Naccache, 2019). The detection time of the mNGS method is short, and its application in the diagnosis of pulmonary infection, pediatric BSI, and intracranial infection has been reported in various studies (Hu et al., 2021; Liu et al., 2021; Nan et al., 2022). However, few reports on its application in adult sepsis and BSI are available. In this study, we discussed the clinical

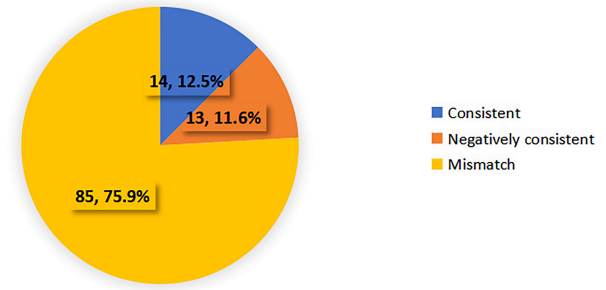

FIGURE 4
Consistency analysis of pathogens detected by blood culture and mNGS.

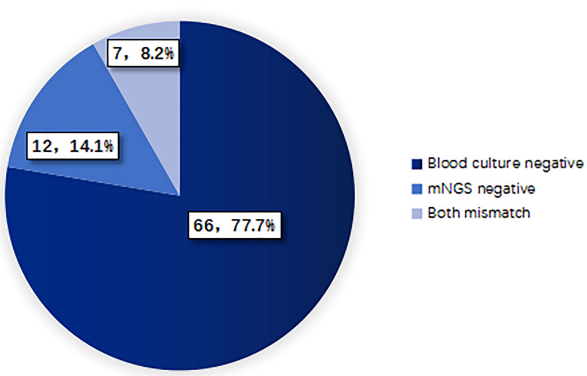

FIGURE 5
Inconsistency analysis of pathogens detected by blood culture and mNGS.

implications and value of mNGS as a new pathogen detection tool for adult sepsis and BSI.

A total of 194 patients with sepsis and BSI were included in this study. More than half of the patients received mechanical ventilation and vasoactive drug therapy, with high APACHE II and SOFA scores. The four most commonly detected pathogens by mNGS and blood culture method were *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Escherichia coli*, and *Pseudomonas aeruginosa*, indicating that the common infectious pathogen in ICU is still Gram-negative bacillus, which is consistent with the current prevalence of hospital-related infections.

In a study where mNGS was applied to guide the treatment of 178 patients with severe pneumonia, the patients' 28-day and 90-day survival rates improved significantly (Xie et al., 2019). A retrospective study of mNGS application in the clinical diagnosis and prognosis of acute respiratory distress syndrome in severe pneumonia showed that the 28-day mortality rate of the mNGS group was significantly lower than that of the non-mNGS group (Zhang et al., 2020). The results from our study, which were consistent with those of (Zhang et al., 2020), showed that the 28-day mortality rate of the mNGS group was significantly lower than that of the non-mNGS group. The total hospitalization time in the mNGS group was longer than that in the non-mNGS group, but the differences in ICU hospitalization time, mechanical ventilation time, vasoactive drug use time, and 90-day mortality rate between the two groups were not statistically significant. This indicated that the patients with sepsis and BSI who underwent blood culture and mNGS are afflicted with the disease for a longer time and have a lower mortality in the short term. This may be due to the earlier adjustment of antibiotic therapy in patients in the mNGS group, which resulted in a higher 28-day survival rate. In contrast, the difference between the 90-day mortality rate in the two groups was not statistically different. Mortality rate is affected by various factors, and further randomized controlled prospective studies are needed to rule out the effect of confounding factors on mortality.

TABLE 2 Clinical medication guidance between mNGS group and non-mNGS group.

	mNGS group (n = 112)	Non-mNGS group (n = 82)	p
Adjustment times of antibiotics	2.13 ± 1.15	1.73 ± 1.40	0.060
Antibiotics used before blood culture	51 (45.54%)	29 (35.37%)	0.185
APACHE II score 7 days after the first adjustment of antibiotics	14.60 ± 6.43	16.00 ± 6.35	0.346
SOFA score 7 days after the first adjustment of antibiotics	7.60 ± 4.21	7.07 ± 4.12	0.809

Reports have shown that the sensitivity and positive predictive value of mNGS for bacterial detection are as high as 97.1% and 94.1%, respectively, which is significantly higher than those of traditional bacterial and fungal smears, cultures, inflammatory markers (Fang et al., 2020). A prospective, observational, single-center study of 256 plasma samples from 48 septic patients showed a positive blood culture rate of 11% and a positive mNGS rate of 71% (Grumaz et al., 2019). In a study of critically ill patients suspected of BSI, it was found that the positive rate of mNGS was 68.3% (excluding viruses), and the positive rate of blood culture was 16.7% (Hu et al., 2021). The high positive rate of blood culture in our study and the positive rate of mNGS are comparable to existing literature, which may be related to the fact that 58.8% of the patients were not treated with antibiotics before blood culture.

Pathogens identified by mNGS and blood culture were also compared. The types of bacteria, especially Gram-negative bacilli, detected by blood culture were identical to those of mNGS. The two methods detected the most Gram-negative bacilli, and the sequences were essentially the same, proving that Gram-negative bacilli account for the majority of sepsis and BSI in the ICU. However, in terms of fungi, the blood culture test detected more of the candida species, while the mNGS test detected *Pneumocystis jirovecii* and *Rhizomucor miniaturus*. Relevant research has shown that *Pneumocystis* is almost entirely non-existent in healthy human lungs, but *Pneumocystis* fragments can infiltrate the peripheral blood through respiratory tract infection sites, especially in the case of immunosuppression (Zhang et al., 2019; Geng et al., 2021). Therefore, the detection of fungi commonly found in the lungs by mNGS in peripheral blood has limited value in diagnosing BSI.

TABLE 3 Comparison of primary and secondary outcomes in early, intermediate, and late groups.

	Early group (*n* = 47)	Intermediate group (*n* = 35)	Late group (*n* = 30)	*p*
Antibiotics used before blood culture	22 (46.81%)	19 (54.29%)	10 (33.33%)	0.233
Total hospitalization time (d)	10 (6, 26)	19 (10, 34)	30 (18, 43)	0.002
ICU Hospitalization time (d)	6 (2, 10)	6 (3, 15)	17 (6, 31)	0.002
28-day mortality	33 (70.21%)	16 (45.71%)	9 (30.00%)	0.002
90-day mortality	33 (70.21%)	20 (57.14%)	13 (43.33%)	0.063
Mechanical ventilation time (h)	27 (1, 56)	18 (0, 179)	119 (0, 297)	0.666
Vasoactive drug use time (h)	28 (2, 78)	10 (0, 96)	30 (0, 239)	0.647

Low positive rates of mNGS for fungal detection in sepsis patients have been reported (Long et al., 2016; Grumaz et al., 2019). Our study contains similar findings, suggesting that mNGS has limited utility in fungal BSI. Blood cultures failed to detect viruses, unlike mNGS, which not only detected viruses but also detected rare pathogens (*Leishmania donovani* species complex, *Bacillus cereus* population) and was therefore more suitable to detect unknown infections, a finding which is consistent with previous studies (Duan et al., 2021; Geng et al., 2021; Govender et al., 2021; Zhan et al., 2022).

In addition, consistency analysis of mNGS and blood cultures showed that 24.1% of pathogens identified in the mNGS group were consistent with blood cultures, and 75.9% were inconsistent with blood cultures. In the absence of agreement, 77.7% was negative for blood cultures, and 14.1% was negative for mNGS. It indicated that mNGS plays an important role in patients with negative blood cultures and provides a reference for the adjustment of antibiotic therapy in those patients. The use of mNGS for pneumonia pathogen identification was described in a large, multi-center prospective study of 159 patients, resulting in treatment change in 59 patients (37.1%), 40 of which (25.2%) received effective antibiotic treatment while avoiding unnecessary antibiotic use (Zhou et al., 2021). In terms of clinical medication guidance, we found that mNGS has become an important reference for the adjustment of antibiotics in sepsis and BSI in the ICU. However, the adjustment of the first antibiotic treatment according to mNGS did not decrease the severity of the disease in the short term.

The optimal time window for mNGS detection in BSI has yet to be determined due to the lack of studies on the impact of antibiotic therapy on the diagnostic performance of mNGS. This study suggested that the positive rate of mNGS could still be higher than that of culture 1 to 2 weeks after the disease onset (Brenner et al., 2018). Over time, blood culture and mNGS had complementary advantages. Therefore, it is currently recommended that mNGS be performed as soon as possible after the diagnosis of disease in patients. If conditions allow, while blood culture samples are taken and stored at -16 to -20°C, blood samples can be taken for subsequent mNGS. After sub-group analysis, it was found that the total hospitalization time in the late group was longer than that in the early group, and the ICU hospitalization time in the late group was longer than that in the early and intermediate groups, suggesting that the early application of mNGS might shorten the ICU hospitalization time and the total hospitalization time. The 28-day mortality rate was higher in the early group, which might be due to the baseline data. The patients in the early group were older and were in more critical condition. The laboratory results suggested more severe organ damage. Although there was no statistical difference, multiple factors might contribute to the increased mortality rate.

Our study contained a few limitations. Firstly, only the top four pathogens in the number of detection sequences and their relative abundance were considered among the pathogens detected by mNGS. Therefore, other major pathogens might have been missed, resulting in the underestimation of the detected pathogens. Secondly, our research did not study the impact of conducting mNGS on hospital expenses such as hospitalization expenses and ICU hospitalization expenses and the effect it had on the patient's economic status. Thirdly, propensity score matching was not carried out to rule out confounding variables due to the incomplete comparison of baseline data, which may have had some effect on the mortality rate.

In summary, compared with traditional blood culture methods, mNGS has certain advantages in the diagnosis of pathogens causing BSI and, subsequently, sepsis. In the early stage of sepsis and BSI, simultaneous detection of pathogens using mNGS technology can lead to early adjustments in antibiotic treatment and reduce the mortality rate. It is an advisable early pathogen detection method for sepsis and BSI. The earlier mNGS is performed, the shorter the ICU hospitalization time and the total hospitalization time will be. Future prospective randomized controlled studies with large sampling sizes are needed to comprehensively assess the diagnostic value of mNGS for sepsis and BSI. Moreover, for BSI, the high sequence number and relative abundance of pathogens detected by mNGS as a diagnostic threshold need to be further determined by large-scale clinical studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committee of Scientific Research and Clinical Trials of the First Affiliated Hospital of Zhengzhou University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YCZ designed the scheme, CQ and SZ were in charge of patient management, FY collected data, and YYZ and XD revised this article.

All authors contributed to the article and approved the submitted version.

Acknowledgments

All authors appreciate the valuable comments from the editor and reviewers of *Frontiers in Cellular and Infection Microbiology*.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1117987/full#supplementary-material

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TYPE Original Research
PUBLISHED 20 February 2023
DOI 10.3389/fcimb.2023.1130483

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 23 December 2022
ACCEPTED 09 February 2023
PUBLISHED 20 February 2023

CITATION
Liu G, Wang L, Li X, Zhang Y, Long H, Wang Y, Gao H, Xia H and Qian S (2023) The value of next-generation metagenomic sequencing in pathogen detection of pleural effusions and ascites from children with sepsis.
Front. Cell. Infect. Microbiol. 13:1130483.
doi: 10.3389/fcimb.2023.1130483

The value of next-generation metagenomic sequencing in pathogen detection of pleural effusions and ascites from children with sepsis

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Objective: To investigate the diagnostic value of metagenomic next-generation sequencing (mNGS) using pleural effusion and ascites from children with sepsis.

Methods: In this study, children with sepsis or severe sepsis and appeared pleural or peritoneal effusions were enrolled, of whom the pleural effusions or ascites and blood samples were conducted pathogen detection using both conventional and mNGS methods. The samples were divided into pathogen-consistent and pathogen-inconsistent groups based on the consistency of mNGS results from different sample types, and into exudate and transudate groups based on their pleural effusion and ascites properties. The pathogen positive rates, pathogen spectrum, consistency between different sample types, and clinical diagnosis consistency were compared between mNGS and conventional pathogen tests.

Results: A total of 42 pleural effusions or ascites and 50 other type samples were collected from 32 children. The pathogen positive rate of the mNGS test was significantly higher than that of traditional methods (78.57% *vs*. 14.29%, $P < 0.001$) in pleural effusion and ascites samples, with a consistent rate of 66.67% between the two methods. Nearly 78.79% (26/33) of mNGS positive results of the pleural effusions and ascites samples were consistent with clinical evaluation, and 81.82% (27/33) of these positive samples reported 1-3 pathogens. The pathogen-consistent group outperformed the pathogen-inconsistent group in terms of consistency with respect to clinical evaluation (88.46% *vs*. 57.14%, $P = 0.093$), while there was no significant difference between the exudate and transudate groups (66.67% *vs*. 50.00%, $P = 0.483$).

Conclusion: Compared to conventional methods, mNGS has great advantages in pathogen detection of pleural effusion and ascites samples. Moreover, consistent results of mNGS tests with different sample types provide more reference values in clinical diagnosis.

KEYWORDS

metagenomic next-generation sequencing (mNGS), pathogen detection, children, pleural effusions and ascites, sepsis

Background

Sepsis has a high morbidity and mortality in the pediatric population, and effective anti-infective treatment is crucial to increase the survival rate (Fleischmann-Struzek et al., 2018). However, traditional techniques for pathogen detection are often time-consuming and have low positive rates, leading to additional reliance on experience in identifying infections and targeted anti-infectious therapy. Metagenomics next generation sequencing (mNGS) test is rapid, highly sensitive, and less affected by previous treatment. Although there are still some drawbacks, it has been shown to have significant advantages over conventional methods in the detection of body fluids such as blood, sputum, and cerebrospinal fluid (CSF) (Miller et al., 2019; Zinter et al., 2019).

Sepsis tends to develop more quickly in children than in adults. Therefore, conducting rapid and comprehensive pathogen screening of biological samples from a variety of suspected infection foci, such as cerebral fluid, pleural effusion, or ascites, is a highly important method to improve the accuracy of pathogen diagnosis and reduce the fatality rate. Currently, mNGS pathogen detection technique offers the possibility to realize this clinical demand. Children with sepsis in pediatric intensive care unit (PICU) are mostly complicated with underlying diseases and multiple organ dysfunction. Pleural or peritoneal effusions are common symptom of infectious diseases in children (Karnsakul et al., 2017) and can lead to a poor prognosis and even death (Piotrowski et al., 2006; Khan et al., 2009; Kookoolis et al., 2014). The formation mechanism of pleural and peritoneal effusion is complex, and whether it is secondary to infection is difficult to determine, and the detection of causative agents remains the gold standard. Traditional pathogen detection methods are far from meeting clinical needs due to their limitations (Huang et al., 2020). Though mNGS is an increasingly emerging and relatively ideal auxiliary detection method, the value of mNGS for pathogen detection in pleural and peritoneal effusions in children with sepsis are not completely understood due to the small number of relevant studies.

In this study, we collected pleural and peritoneal effusion samples as well as other sample types simultaneously from 32 children with sepsis and hospitalized in PICU.

For the first time, this study described the positive rate of mNGS pathogen detection in pleural effusions and ascites samples of children with sepsis, and pathogen consistency and clinical consistency were also compared with those of other samples, aiming at providing valuable evidence for the values of clinical mNGS pathogen diagnosis in pleural and abdominal fluid samples.

Materials and methods

Samples and data collection

Children with sepsis or severe sepsis who underwent mNGS test of pleural or peritoneal effusions in the PICU of Beijing Children's Hospital were enrolled. The inclusion criteria: 1) met the diagnostic criteria for sepsis and severe sepsis as defined by the 2005 International Sepsis Guidelines for Children (Goldstein et al., 2005); 2) aged between 29 days and 18 years old; 3) hospitalized at the PICU of Beijing Children's Hospital; 4) had pleural or peritoneal effusion samples collected for pathogen detection by mNGS; 5) underwent etiological examination by other methods such as culture of samples from the same site at the same time, or underwent mNGS test of the body fluid samples collected from different sites at the same time. The exclusion criteria: 1) had only pleural or peritoneal effusions but no additional site specimens sent for examination in the same pathogen screening by mNGS detection; 2) had only mNGS test but no traditional test results for the same biological sample; 3) had only electronic case records of mNGS results but no original reports; 4) had incomplete clinical data. This study was a secondary analysis based on the data of a prospective study of severe sepsis in children (2021.11~2023.12) (Beijing Children's Hospital Ethical Approval No. [2022] -E-019-Y). mNGS results of pleural or peritoneal effusion samples from a total of 45 pediatric patients were collected, from which 32 children who met the inclusion and exclusion criteria were selected. Clinical data, including demographic information, organ dysfunction, underlying disease, laboratory tests, results of conventional and mNGS tests, and in-hospital mortality, were collected in this study.

Grouping method

Patients were divided into exudate and transudate groups based on the original state of their pleural and peritoneal effusions. Pleural effusion was judged to be exudate if it met at least one item of the Light criteria, otherwise it was judged to be transudate. The Light

criteria (Light et al., 1972) are total protein in pleural fluid/total protein in serum > 0.5, LDH in pleural fluid/LDH in serum > 0.6 and LDH activity in pleural fluid ≥ 200 U/L. Peritoneal effusion was judged as exudate if it met at least two items of Boyer's criteria, otherwise it was judged as transudate. The Boyer's criteria (Boyer et al., 1978) are total protein in ascitic fluid/total protein in serum ≥ 0.5, LDH in ascitic fluid/LDH in serum ≥ 0.6 and LDH activity in pleural fluid ≥ 400 U/L.

Clinical coincidence: if at least one pathogen in the mNGS results of pleural or peritoneal effusion samples conformed to the clinical judgment, the current diagnosis and treatment plan were adjusted or confirmed according to the positive or negative results, it was judged as a clinical coincidence, and vice versa.

If a same microorganism is positive in pleural effusions or ascites and other sample types collected at the same time, it's more likely to be the pathogenic pathogen, which can improve the pathogen diagnosis accuracy. In this study, pathogen-consistent and pathogen-inconsistent groups were set to further clarify whether the detection of the same pathogen can benefit the children more. If at least one pathogen in the mNGS results of pleural or peritoneal effusions was consistent with the pathogen detected in other samples, it was judged to be pathogen-consistent, and vice versa.

mNGS pathogen detection

The clinical samples were performed PACEseq mNGS test by Hugobiotech Co., Ltd. During the experimental process, negative controls (sterile deionized water) and positive controls (synthesize fragments with known quantities) were established for each batch of experiments using the same wet lab procedures and bioinformatics analysis as the clinical samples. The read number and reads per million (RPM) of each detected microbe were calculated. For detected microbes, including bacteria (*Mycobacterium* excluded), fungi (*Cryptococcus* excluded), and parasites, a positive mNGS result was given when its coverage ranked in the top 10 of similar microbial species (or genera) and was absent in the negative control ("No template" control, NTC) or when its ratio of RPM between sample and NTC (RPMsample/RPMNTC) > 10 if RPMNTC≠0. For viruses, *Mycobacterium*, and *Cryptococcus*, a positive mNGS result was considered when at least one unique read was mapped to species level and absent in NTC or RPM sample/RPMNTC > 5 when RPMNTC≠0.

Statistical analysis

Statistical analysis was performed using SPSS 24.0 statistical software. Measurement data in accordance with normal distribution were expressed as X ± s, and one-way analysis of variance was used for comparison between groups. Measurement data in the non-normal distributions were expressed as median (interquartile range) [M (P25, P75)], and the Mann-Whitney U test was used for comparison between groups. Enumeration data were expressed as percentages (%), and $\chi 2$ test or Fisher exact test was used for comparison between groups. $P<0.05$ was considered statistically significant.

Results

Clinical features and mNGS samples

A total of 32 pediatric patients with sepsis were included in the study (Figure 1A). The average age was 7 (3, 10) years old, and 59.38% (19/32) were male. Their major organ dysfunctions were respiratory insufficiency (53.13%), acute kidney injury (46.88%), and septic shock (34.38%). The medians of procalcitonin (PCT) and C-reaction protein (CRP) levels were 16.0 (1.5, 58.5) ug/L and 14.0 (1.8, 80.5) mg/L, respectively. 81.25% (26/32) of the patients had underlying diseases, with tumors accounting for 50.00% (13/26). In addition, the in-hospital mortality was 18.75% (6/32) (Tables 1, S1).

A total of 42 pleural effusions or ascites samples were obtained in this study. Four patients simultaneously collected pleural

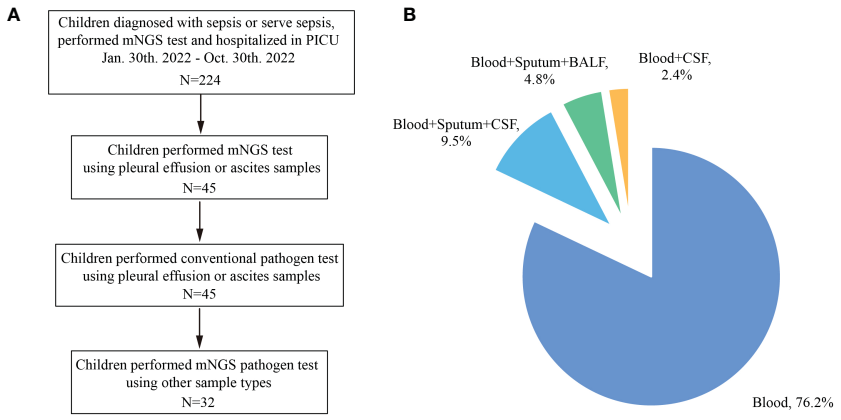

FIGURE 1
Sampling strategy. **(A)** flow graph showing the patient screening procedures; **(B)** pie graph showing the performed mNGS test of included patients.

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TABLE 1 Clinical features and laboratory tests of involved patients (N=32).

Clinical features	Values
Age [years, M (Q1, Q3)]	7 (3, 10)
Gender [male, %]	19 (59.4)
Organ dysfunction [case, %]	
Respiratory insufficiency/failure	17 (53.13)
Acute kidney injury	15 (46.88)
Septic shock	11 (34.38)
Cardiac insufficiency/failure	9 (28.13)
Brain dysfunction	7 (21.88)
Gastrointestinal failure	2 (6.25)
Hepatic failure	1 (3.12)
Disseminated coagulation disorders	1 (3.12)
Laboratory tests [M (Q1, Q3)]	
PCT [μg/L, M (Q1, Q3)]	16.0 (1.5, 58.5)
WBC [$\times10^9$/L, M (Q1, Q3)]	6.4 (4.0,12.9)
CRP [mg/L, M (Q1, Q3)]	14.0 (1.8, 80.5)
Underlying disease [case, %]	26 (81.25)
Tumor	13 (40.63)
Malnutrition	8 (25.00)
Low immunity	6 (18.75)
Others [a]	4 (12.5)
In-hospital death [case, %]	6 (18.75)

[a]Others included one case each of lysosomiasis, dermatomyositis, Langerhans cell histiocytosis, and congenital neutropenia.

effusions and ascites samples. Four patients had two, and one patient had three ascites samples collected. Any pleural effusion or ascites sample was regarded as an independent test. Among them, 76.19% (32/42) were detected only in combination with blood samples, 9.52% (4/42) were detected in combination with blood, deep sputum, and CSF, 4.76% (2/42) were detected in combination with blood, deep sputum, and bronchoalveolar lavage fluid (BALF), and 2.38% (1/42) were detected in combination with blood and CSF (Figure 1B; Table S2).

Positive rates of conventional and mNGS tests

The positive rates of pathogens in pleural or peritoneal effusions, blood, sputum, CSF and other body fluids by the conventional method were 14.29% (6/42), 11.11% (4/36), 20.00% (1/5), 0 and 0, respectively. However, the positive rates by mNGS were 78.57% (33/42), 83.33% (30/36), 100.00% (5/5), 40.00% (2/5) and 75.00% (3/4), respectively, which were great higher than those of the conventional test ($P < 0.05$) (Figure 2A). Furthermore, mNGS detected more pathogens at a single test in comparison to the conventional methods. Only 3.26% (3/92) of the conventional tests returned results for multi-pathogens. The proportions of 1-3 pathogens detected in pleural and peritoneal effusion, blood, deep sputum, and other body fluid were 64.29% (27/42), 72.22% (26/36), 80.00% (4/5), 40.00% (2/5) and 50.00% (2/4), respectively, while the proportions of 4-6 pathogens detected were 14.28 (6/42), 11.11% (4/36), 20.00% (1/5), 0 and 25.00% (1/4) (Figure 2B).

Consistency between conventional and mNGS tests

Six (14.29%, 6/42) pleural effusion or ascites samples were tested positive by traditional culture, 66.67% (4/6) of which were consistent with mNGS results. Four blood and one deep sputum samples were tested positive by traditional culture, and the consistency rate with mNGS results was 100% (4/4, 1/1). The consistencies of mNGS results between pleural effusions or ascites and other samples are shown in Figure 3. The proportion of the same pathogens detected in pleural effusions or ascites as in blood,

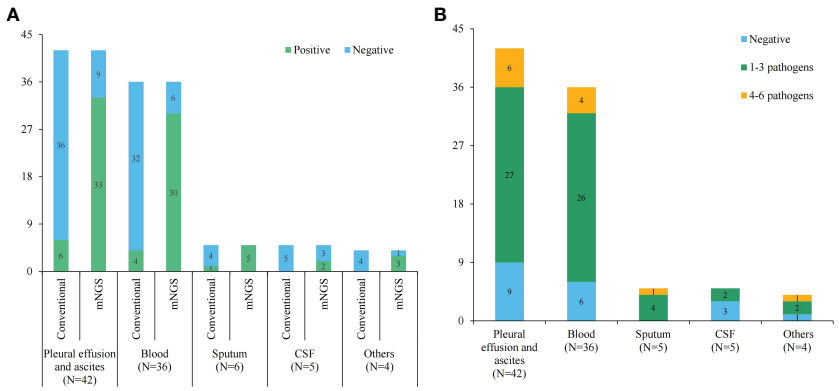

FIGURE 2
The statistics of conventional and mNGS pathogen tests. **(A)** The positive and negative rates of conventional and mNGS pathogen tests in different sample types; **(B)** histogram showing detected pathogens of mNGS test in different sample types.

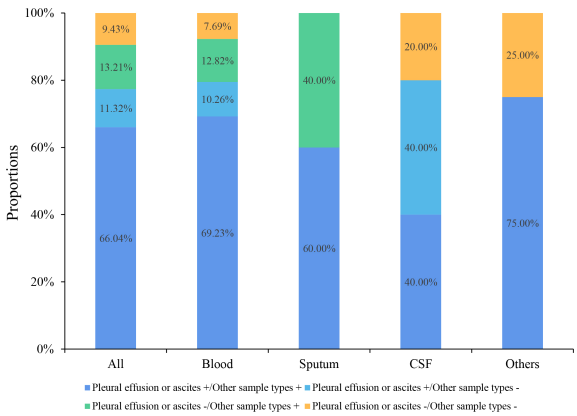

FIGURE 3
Consistency of mNGS test results between pleural effusions or ascites and the other sample types.

deep sputum, bronchoalveolar lavage fluid, and other body fluid samples were 69.23% (27/39), 60.00% (3/5), 40.00% (2/5), and 75.00% (3/4), respectively. A total of 11 G- bacteria, 5 G+ bacteria, 2 fungi and 5 viruses were simultaneously detected in blood and other sample types. It's worth noting that 8 G- bacteria, 3 G+ strains, 2 fungi and 3 viruses were only detected in pleural or peritoneal effusions (Figure 4).

Clinical consistency between the pathogen-consistent and pathogen-inconsistent groups

Most of the patients were collected with one set of samples. Four patients with two sets of samples and one patient with three sets of samples were grouped into pathogen-consistent group. A patient with two sets of data from were separated into two groups. The PCT (16 *vs.* 2.3, $P = 0.141$) and CRP (14 *vs.* 11, $P = 0.401$) levels were both higher in the pathogen-consistent group than in the pathogen-inconsistent group but showed no significance. The pathogens detected in 27 pleural effusions or ascites samples were consistent with clinical findings, suggesting that infectious pleural or peritoneal effusion samples accounted for 64.29% (27/42). Among them, 85.2% (23/27) of the detected pathogens in the pathogen-consistent group were consistent with the clinical judgment, which was higher than it (57.0%) in the pathogen-inconsistent group ($P = 0.093$). There was no significant difference in mortality and adjustment rate of antibiotic therapies between the two groups (Table 2).

Clinical coincidence of mNGS tests in exudate and transudate groups

The positive rate of bacteria was observed great higher in the exudate group than in the transudate group (70.0% *vs.* 41.7%, $P = 0.158$) than those in the transudate group. At the same time, the mean specific reads number (130 *vs.* 3, $P = 0.009$) were also discovered significant higher in the exudate group. In addition, the coincidence between detected pathogens and clinical findings was also higher in the exudate group, but no statistically significant difference was observed (66.67% *vs.* 50.00%, $P = 0.483$) (Table 3).

Discussion

Pleural or peritoneal effusions are common complicated symptoms. The causes of pleural and peritoneal effusions in children are complex and can be primary or secondary infections or a variety of non-infectious diseases such as liver diseases, heart diseases, and tumors (Hildreth et al., 2009; Giefer et al., 2011). Previous studies have found that approximately 14.9% of peritoneal effusions in hospitalized children were associated with infection (Karnsakul et al., 2017). Utine et al. reported that among 492 hospitalized children with pleural effusion, the rate of secondary infection with pleural effusion was up to 77.4% (Utine et al., 2009). But for a long time, clinicians have placed additional emphasis on the therapeutic value of effusion drainage, as well as its diagnostic value for tumors or immunological diseases. The significance of pleural or peritoneal effusions in etiological diagnosis has not been completely realized because of the poor positive rate of conventional pathogenic tests. Although mNGS offer considerable promise for the detection of potential pathogens in such biological samples, there haven't been any relevant reports on the clinical significance of their etiological diagnostics.

Serum PCT level is related to the severity of infection (Bartoletti et al., 2018) and has a certain predictive effect on thoracoabdominal infection (Lin et al., 2009; Su et al., 2013). In this study, the median serum PCT level was as high as 16.0 μg/L, and 34.3% patients had septic shock, which indicated that the possibility of severe infection or pleural and peritoneal effusion superimposed with infection was high in this group of children. Tumor is a common underlying disease of sepsis in PICU. Due to the weak immune barrier function, it is easy to lead to multi-site or multi-pathogen infection and poor prognosis. In this study, about 81.25% of the children had underlying diseases, of which tumors accounted for 50.00%. Therefore, identifying the pathogen as soon as possible is the key to improve the prognosis.

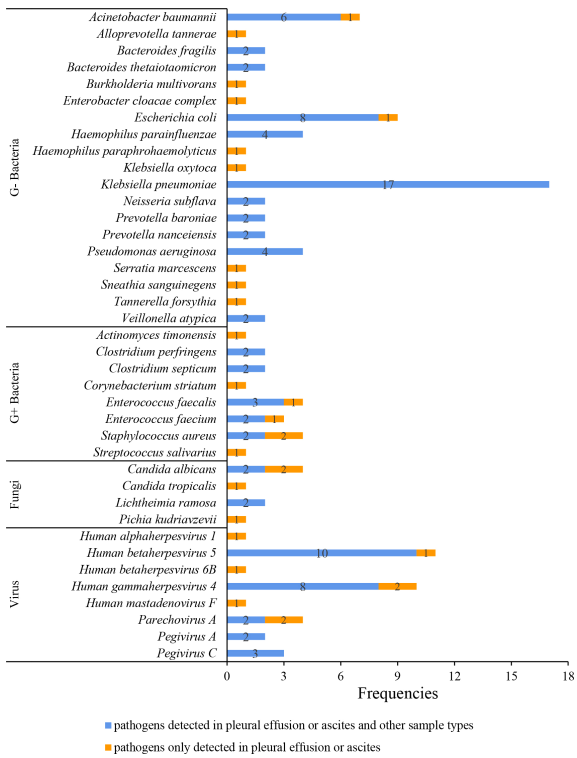

FIGURE 4
Pathogen spectrum detected by mNGS in different sample types.

To improve the pathogen positive rate and pathogen diagnosis accuracy, clinicians can collect samples from multiple suspected infected sites for mNGS pathogen screening (Chien et al., 2022). As one of the most common sample types, blood has the easiest access for mNGS testing and is the most important sample type for joint analysis in this study. It was reported that the positive rate of mNGS test of blood samples in critically ill adult patients hospitalized in ICU was 5~6 times that of conventional methods (41.3% *vs.* 7.9%), and co-infection could be identified (Geng et al., 2021). Similarly, blood mNGS tests in children with suspected sepsis in PICUs can detect a variety of pathogens (Yan et al., 2021). In this study, the positive rate of mNGS test in blood samples was nearly eight times that of traditional tests (83.33% *vs.* 11.11%), and the proportions of 1-3 and 4-6 pathogens detected were 72.22% and 11.11%, respectively. It was reported that the positive rate of mNGS test in blood samples of adult patients with sepsis was 62.0% (Chien et al., 2022), while a higher positive rate (83.33%) of children with sepsis was observed in this study. However, no mNGS related

TABLE 2 Comparison of clinical features and pathogen constitution between the pathogen-consistent group and pathogen-inconsistent group.

Test results	Pathogen inconsistent group (N=6)	Pathogen consistent group (N=27)	X^2/ Z	P value
PCT [μg/L, M (Q1, Q3)]	2.3 (1.1, 29)	16 (4.3, 79)	1.472	0.141
CRP [mg/L, M (Q1, Q3)]	11 (2.7, 27.5)	14 (0.7, 91)	0.841	0.401
WBC in pleural and ascitic fluid [$\times10^9$/L, M (Q1, Q3)]	1,377.5 (472.3, 2,788.3)	553 (186, 3,577)	0.25	0.803
Neutrophil ratio in pleural and ascitic fluid [%, M (Q1, Q3)]	53.5 (12.5, 82.5)	45 (27,84)	0.15	0.881
Total protein in pleural and ascitic fluid [g/L, M (Q1, Q3)]	27.1 (23.0, 30.0)	29.3 (23.2, 38.3)	0.575	0.565
Pleural and ascitic fluid test results consistent with clinical findings [case, %]	3 (50.0)	23 (85.2)	-	0.093[a]
add new anti-infective drugs	4 (100)	11 (47.8)	-	0.484[a]
reduce anti-infective drugs	0 (0)	6 (26.1)	-	0.16[a]
remain unchanged	0 (0)	6 (26.1)	-	0.16[a]
In-hospital mortality [b]	2 (33.3, 2/6)	3 (15.0, 3/20)	-	0.216[a]

[a]Fisher's test; [b]Mortality comparison was calculated in line with the first time mNGS test in patient with multi-time mNGS tests.

TABLE 3 Comparison of some observation indicators of pleural and peritoneal effusion exudate and transudate.

Results	Exudate group (N=30 samples)	Transudate group (N=12 samples)	Z value	P value
Bacteria [sample, %]	21 (70.00)	5 (41.67)	–	0.158[c]
Virus [sample, %]	7 (23.33)	7 (58.33)	–	0.67[c]
Fungi [sample, %]	4 (13.33)	1 (8.33)	–	1.000[c]
Negative [sample, %]	5 (16.67)	4 (33.33)	–	0.406[c]
Mean specific sequence number [reads, M (Q1, Q3)]	130 (13, 4,047) [a]	3 (1, 3.5)[b]	2.606	0.009
Consistence with clinical findings [sample, %]	20 (66.67)	6 (50.00)	–	0.483[c]

[a]refers to 35 pathogens; [b]refers to 19 pathogens; [c]refers to Fisher's test.

studies of pleural or peritoneal effusions have been reported so far. This study showed that the positive rate of mNGS test in pleural effusions and ascites from children with sepsis was 78.8%, which was great higher than it (14.3%) in traditional culture. Furthermore, multi-type pathogens were detected by a single test in 42.86% (18/42) samples. Previous studies have shown that mNGS test has higher accuracy and specificity than the conventional methods (Gu et al., 2021), and this study showed a 66.7% agreement rate between the two methods in the regular pathogenic tested positive samples. It can be concluded that mNGS test is much more sensitive than conventional tests and can provide more comprehensive results in the pathogenic detection of pleural effusion and ascites samples. However, due to the technical limitations such as influence cause by background microorganisms in sampling and experimental progress, higher positive rates are not always better. The detection of pathogens in accordance with the clinician's judgment is of the utmost concern. In this study, 78.79% of mNGS tests returned pathogens consistent with clinical judgments, a small number of potential false positives due to technical limitations were acceptable.

Generally, the same pathogen detected in samples from multiple sites or multiple samples from the same site is considered more likely to be the pathogen responsible for the infection. In this study, we discovered that the proportion of the same pathogen detected in pleural effusion or ascites and blood was relatively high (69.23%), which not only indicated that the infectious foci in pleural effusions or ascites and blood may be homologous, but also suggested that the results of blood mNGS detection had a good reference value for judging whether pleural and peritoneal effusion was secondary infection in children with sepsis. Judging from the types of pathogens detected, G- bacteria are predominant, with *Klebsiella pneumoniae* being the most common. *Klebsiella pneumoniae* is a normal colonized bacteria that present in human gut and respiratory tract. Factors such as infection, intestinal microecological imbalance, and immune barrier disruption can cause conditional pathogenesis of *Klebsiella pneumoniae*. Previous studies have shown that *Klebsiella pneumoniae*-related sepsis can significantly increase the risk of systemic multi-site infections (Li et al., 2021), and thoracoabdominal membranes are the potentially affected tissues (Ren et al., 2020; Ameer et al., 2022). Interestingly, *Klebsiella pneumoniae* was only detected in multiple samples at the same time. However, some pathogens detected in pleural effusions or ascites samples by mNGS were completely different from those in any other samples. For example, eighteen pathogenic organisms were only positive in pleural effusions or ascites, inferring that they might originate from adjacent foci of infection such as the lungs or digestive tract. Besides, 15.4% and 16.7% of the blood and deep sputum samples were negative, but the corresponding pleural effusions or ascites were positive. In spite of only common pathogens were detected in this study, some rare and diagnostic pathogens could also be detected in pleural effusions or ascites (Gupta et al., 2018). Thus, the results of mNGS should be carefully analyzed in combination with clinical conditions. In addition, mNGS is highly sensitive to both cytomegalovirus and Epstein-Barr virus (Duan et al., 2021), and this study also showed certain positive rates for pleural effusions or ascites.

To further investigate the clinical significance of the mNGS results in pleural effusions and ascites, the clinical characteristics and pathogen composition of each group were analyzed in multiple groups and compared in this study. First, samples were divided into pathogen-consistent and pathogen-inconsistent groups according to whether the detected pathogens in pleural effusions or ascites are consistent with the test results of other sample types (Table 2). PCT is a common indicator to determine bacterial infection, and several studies have shown that PCT levels are correlated with the abundance of pathogens detected in the blood of children with suspected sepsis in the PICU (Yan et al., 2021). In this study, the median PCT in the pathogen-consistent group was great higher than that in the pathogen-inconsistent group, which may be related to the relatively high proportion of pathogenic bacteria detected. Furthermore, it's worth noting that the degree of clinical coincidence in pathogen-consistent group is better than it in the pathogen-inconsistent group (85.2% *vs* 50%, $P = 0.093$) as well as the mortality (15.0% *vs*. 33.3%, $P = 0.216$). This suggests that a combined multiple-sample test may have possible advantages in improving clinical diagnosis rates and prognosis, and the consistent pathogens proved by multiple-sample should be placed high priority in clinical practice. Certainly, further studies with larger sample size are needed to verified this view.

In order to determine whether the original character of pleural effusions and ascites could affect the pathogen detection of mNGS test, the pleural effusions and ascites samples were divided into exudate and transudate groups. Although fluid exudations are mostly caused by inflammation, they can also be caused by

various other reasons, such as malignant tumors, connective tissue diseases, and pulmonary embolism (Burgess, 2004). Thus, their suggestive effects on infection are limited. In this study, the exudate group had a higher positive rate of bacterial infections than the transudate group, suggesting that bacterial infection is still the primary cause of pleural or peritoneal fluid exudation in this group of children. In addition, the clinical coincidences of these two groups were similar (73.3% and 67.7%), indicating that mNGS results have good clinical value regardless of exudations or transudations.

Conclusions

In this study, we analyzed mNGS tests of pleural effusions and ascites from 32 cases of children with sepsis or severe sepsis, and a high positive rate of mNGS tests was observed in pleural effusions and ascites. mNGS can improve diagnostic accuracy and particular attention should be paid to interpreting the clinical significance of pathogenic organisms with high specific sequence numbers. mNGS test using contemporaneous samples from multiple sites can help improve clinical diagnostics, and pathogens consistent in different sample types have important clinical significance. However, the identification of exudation or transudation alone is of limited value in determining thoracic and abdominal infections. Since this study is retrospective and the sample size is small, potential case selection bias may have some impact on the findings, and further sample size augmentation is required in a future study.

Data availability statement

The datasets presented in this study can be found in National Genomics Data Center (NGDC) (https://ngdc.cncb.ac.cn/) with the project number of PRJCA013799.

Ethics statement

The studies involving human participants were reviewed and approved by the Clinical Research Ethics Committee of Beijing Children's Hospital with approval number of [2022]-E-019-Y. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

SQ, GL, and HX contributed to the study design. GL, LW, XL and YZ drafted the manuscript. YW and HG contributed to sample collection. GL, XL and HL contributed to data collection and analysis. SQ and HX revised the manuscript. All authors contributed to the article and approved the submitted version. All authors read and approved the final manuscript.

Funding

This study was supported by Beijing Municipal Science and Technology Commission (No. Z211100002921063) and Clinical application of high throughput sequencing technology in infectious diseases (MTP2022B018).

Conflict of interest

XL, YZ, LH and HX were employed by Hugobiotech Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1130483/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 23 February 2023
DOI 10.3389/fcimb.2023.1145950

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 16 January 2023
ACCEPTED 13 February 2023
PUBLISHED 23 February 2023

CITATION
Chang L, Qiu L, Lei N, Zhou J, Guo R, Gao F, Dong S, Chen M, Wu F and Qin B (2023) Characterization of fecal microbiota in cervical cancer patients associated with tumor stage and prognosis.
Front. Cell. Infect. Microbiol. 13:1145950.
doi: 10.3389/fcimb.2023.1145950

Characterization of fecal microbiota in cervical cancer patients associated with tumor stage and prognosis

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Cervical cancer (CC) is the fourth most frequent malignancy among women worldwide, and its prevention and treatment are evolving rapidly. The gut microbiota has been reported to play a crucial role both in the preservation of homeostasis and the development of cervical cancer. In this study, we collected fecal samples to investigate the microbial signatures in cervical cancer patients compared with healthy controls using 16S rRNA sequencing analysis and metagenomic next-generation sequencing (mNGS) testing. Our findings demonstrated a substantial difference in the gut microbiota composition of cervical cancer patients and healthy controls. The disease and stage were most significantly negatively correlated with *Ruminococcus 2*, which might be considered a potential clinically relevant biomarker. Functions of differential microbiomes were also analyzed, indicating significant differences in metabolisms and biosynthesis between the two groups. These findings demonstrate that patients with cervical cancer have certain species of gut microbiota that are exclusive to them and particular species have the potential to be used in the prognosis of cervical cancer.

KEYWORDS

cervical cancer, fecal microbiota, 16S rRNA sequencing, metagenomic next-generation sequencing, *Ruminococcus 2*

1 Introduction

Cervical cancer poses a major threat to the health of women globally with around 520,000 new cases and 270,000 deaths each year (Siegel et al., 2020). Although the human papillomavirus (HPV) infection is a significant risk factor for cervical cancer, most HPV infections are temporary, with just 10-15% of high-risk infections remaining and 85-90% of

high-risk infections spontaneously resolving. The persisting HPV infection causes precancerous cervical intraepithelial neoplasia (CIN), which further develops into invasive cervical cancer (ICC) (Shulzhenko et al., 2014). Understanding the processes involved in the tumorigenesis of cervical cancer is still very important. In addition, the early diagnosis and prognosis of cervical cancer provide important information for clinical application.

The human body is colonized by a diverse range of commensal and pathogenic microbial communities, including protists, archaea, bacteria, fungi, and viruses (Gilbert et al., 2018). Recent studies have found that gut microbiota is linked to the occurrence and progression of cancerous diseases. For example, common P53 mutations exert carcinogenic effects in the case of gallic acid produced by microorganisms (Kadosh et al., 2020). In the meanwhile, the commensal gut microbiome may functionally interact with the host's genome to provide protective effects by secreting bioactive compounds. In some cases, the secretion and metabolism of estrogen are greatly affected by gut microbial functions (Plottel and Blaser, 2011; Flores et al., 2012). This results in an estrogen-mediated gut-vagina axis (Baker et al., 2017). The interaction between the gut and the distal vaginal mucosa involves certain microorganisms that metabolize estrogen. The collection of this kind of microbiota is known as estrobolome (Plottel and Blaser, 2011). The alterations in the diversity of the gut microbiota may affect how estrogen is metabolized. And estrogen mediates the production and secretion of glycogen in the vaginal epithelium, resulting in high levels of free glycogen that affect the composition of the vaginal microbiota (Mirmonsef et al., 2016). Vaginal flora imbalance is closely related to HPV infection and cervical intraepithelial lesions. At the same time, estrogen itself can act on HPV response elements to change viral gene expression, as well as accelerate the process of HPV infection (Chen et al., 2021; Wang et al., 2021; Zhang et al., 2021b). And it can also play an immunomodulatory role in the tumor microenvironment to promote the occurrence and development of HPV-positive cervical cancer. Thus, the constituents and effects of microbiomes should be investigated to study the related mechanism in certain diseases (Gagnière et al., 2016; Liu et al., 2018).

In cervical cancer, a study compared the gut microbiome composition of five healthy controls and eight patients to establish a strong association between the gut microbiome and cervical cancer (Wang et al., 2019). They found that the distribution of gut microbiota differed between cervical cancer patients and healthy people, and the gut microbiome may provide potential diagnostic biomarkers for cervical cancer, including *Parabacteroides*, *Escherichia Shigella*, and *Roseburia*. Another study also observed different genres in fecal microbiota dynamics between healthy women and patients with early cervical cancer (Kang et al., 2020). They found that in the cancer group, Proteobacteria were significantly more abundant, including *Escherichia*, *Coli-Shigella*, *Rossella*, *Pseudomonas*, *Clostridium pilonicum*, *Pilospirillaceae*, *Yersinia*, and *Vibrio Succinosus*. Although these studies provide some proof that gut microbiota is different between cervical cancer patients and healthy controls, functions and mechanisms have not been further explored yet.

In this study, we collected fecal samples from 13 patients with cervical cancer and 10 healthy controls. The clinical characteristics of all individuals were analyzed. 16S rRNA sequencing analysis and mNGS testing were used to identify the characteristics of gut microbiota to identify and compare the microbiome. Functions and related mechanisms were further conducted. Our results showed that *Ruminococcus 2* was the most significantly different microorganism, which might be considered a potential clinically relevant biomarker.

2 Methods

2.1 Research overview

In this study, patients with cervical cancer and healthy controls who were admitted to the hospital from February 2022 to June 2022 were collected. The choice was taken and the informed permission was signed by the patients and their families. Following admission, the patient's conditions and the progress of the study were fully disclosed to them as well as to their families. The Zhengzhou University First Affiliated Hospital Ethics Committee has authorized this work (2022-KY-1341-002). The eligibility criteria are as follows: (1) Age: 18-72 years old; (2) All patients with cervical cancer were pathologically diagnosed; All healthy controls underwent detailed CT, MRI, and other imaging examinations. (3) All patients had not received pelvic and systemic concurrent chemoradiotherapy before admission, and the main organ functions were normal. (4) None of the participants had antibiotics administration 3 months before samples collection. And the two groups were matched by age and body mass index (BMI).

2.2 Collection of stool samples

Each subject gave a sample of their most recent tail feces between 06:00 and 10:00 AM. Samples were separated into 200mg aliquots and immediately kept at - 80°C after being inactivated at 70°C for 1 hour. Samples that were left at room temperature for more than two hours were discarded.

2.3 16S rRNA sequencing analysis and data processing

NovaSeq 6000 SP Reagent Kit V1.5 (Illumina, USA) was used for PE250 sequencing and QIIME2 was used for preliminary analysis of sequencing data to obtain microbial annotation and other data, and R software (version 4.2.2) was used for statistical analysis.

2.4 Operational taxon clustering and taxonomic annotation

The same number of reads were randomly selected from all samples and classified into operational taxonomic units (OTUs) by the UPARSE pipeline. We gathered every OTU from every sample used in the discovery, validation, and independence phases. The identity threshold was set at 0.97. We carried out taxonomic studies and further investigations of microbial diversity.

2.5 Library construction

Covaris (Woburn, Massachusetts, USA) randomly fragmented 1 μg of genomic DNA. Magnetic beads were used to filter the DNA fragments to an average size of 200–400 bp. The libraries were then built using end-repair, A-tailing, and adapter ligation. Then it was put through a PE100 mode sequence on the BGISEQ-500 platform.

2.6 Statistical analysis

The Wilcoxon rank-sum test, the Kruskal-Wallis test, and Fisher's exact test were used to assess differences between the two groups for non-normal continuous variables and categorical variables, respectively. Statistical analyses were performed using SPSS V.20.0 for Windows (Illinois, USA). The within-sample and between-sample diversity are represented, respectively, by the α and β diversity indices. In the quantitative insights into microbial ecology (QIIME, ver.1.9.1), α-diversity indices were calculated. The amount of differences between different samples was then calculated for the β -diversity analysis using Principal coordinates analysis (PCoA). The research was then aided by MetagenomeSeq analysis, which evaluated the effects of species abundance using linear discriminant analysis (LDA) effect size (LEfSe). Functional predictions of microbial communities were made using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) program. The extent to which environmental factors influence gut microbial changes were analyzed using CCA analysis. In the illustration, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ was defined as statistically significant. In the principal coordinates analysis, aa=p<0.05, ab= p>0.05. Differences with p-values of < 0.05 (two-sided) were considered to indicate statistical significance.

3 Results

3.1 Clinical characteristics of the recruited subjects

A total of 13 patients with cervical cancer (CC) and 10 healthy controls (HCs) were recruited in this study, and the experimental flow chart of this study is summarized (Figure 1). CC group was pathologically identified and new to cervical cancer. To confirm cervical cancer samples of histological kinds, pathological biopsies were performed on all cervical cancer patients. Age and BMI did not significantly differ between the two groups. The demographic and clinical traits of the CC group and HCs group are displayed in Table 1.

3.2 Gut microbial distribution and diversity between patients with cervical cancer and healthy controls

Differences in the gut microbiota between CC and HCs groups were evaluated by the α-diversity and β-diversity. α-diversity is commonly used to measure the richness of species in the ecology of a community and is a comprehensive indicator of species richness and evenness. While β-diversity refers to the species divergence between different environmental communities (Jost, 2007). β-diversity together with α-diversity makes up the overall diversity. The statistical difference in the α-diversity index in the two groups was not significant (Figure 2A). However, there was a tendency for microbial community diversity in the CC group to be lower than that in the HCs group. The average microbial composition diversity of the two groups was compared using the principal coordinates analysis (PCoA) to ascertain β-diversity. The Pearson Distance and Bray-Curtis Distance showed significant

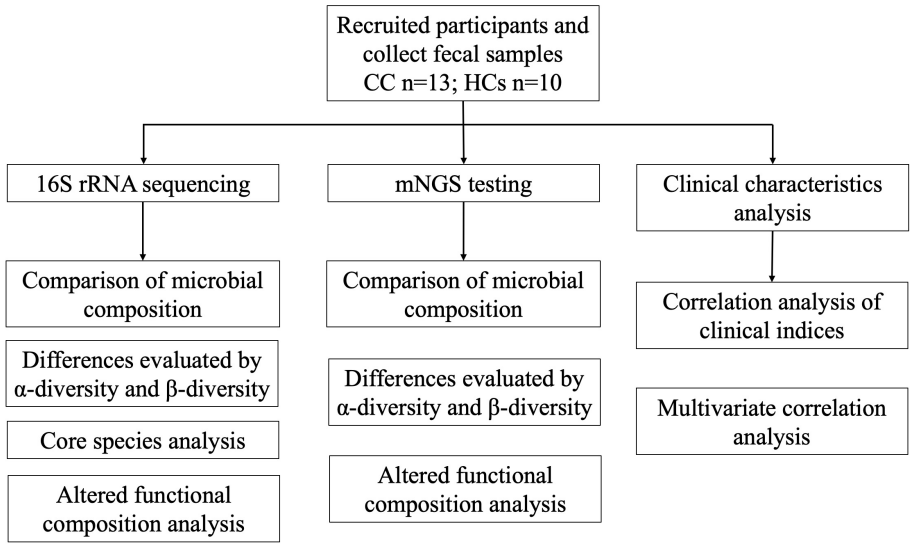

FIGURE 1
Flow chart of the study design. A total of 23 fecal samples were collected, including 13 cervical cancer patients and 10 healthy controls. Analysis in this study is summarized.

TABLE 1 Demographic characteristics of the study participants.

Characteristic	Overall, N = 23	CC, N = 13	HCs, N = 10	*p-value*
Patient Age, Mean	47.09 ± 9.17	48.92 ± 10.81	44.7 ± 6.22	0.284
BMI, Mean	25.11 ± 4.51	24.2 ± 3.19	26.29 ± 5.78	0.282
Gender, n (%)				
Female	23 (100%)	13 (56.4%)	10 (43.6%)	
Stage, n (%)				
I	6 (26.0%)	6 (46.2%)		
II	3 (13.0%)	3 (23.1%)	NA	
III	4 (17.4%)	4 (30.8%)		
Differentiation, n (%)				
High	5 (21.6%)	5 (38.5%)		
In	4 (17.4%)	4 (30.8%)	NA	
Low	4 (17.4%)	4 (30.8%)		
Tumor diameter, n (%)				
≦ 4 cm	9 (39.0%)	9 (69.2%)		
> 4 cm	4 (17.4%)	4 (30.8%)	NA	
Vaginal infiltration, n (%)				
No	10 (43.4%)	10 (76.9%)		
Yes	3 (13.0%)	3 (23.1%)	NA	

differences (aa=p>0.05, ab= p<0.05) in microbial communities between the CC group and the HCs group (Figure 2B). The fecal microbial composition was dominated by *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* at the phylum level (Figure 2C). The genus level (Figure 2D) was dominated by *Bifidobacterium*, *Escherichia Shigella*, *Bacteroides*, and *Blautia*. There was no significant difference in the main flora of gut microflora between the CC group and HCs group at the phylum and genus levels, but there was a difference in the composition ratio of gut microflora. Although α-diversity did not show the difference in the abundance of gut flora, β-diversity indicated the difference between the CC group and HCs group. Combined with the differences at the phylum and genus levels, we observed differences mainly reflected in the species level of gut flora. Species diversity confirmed that gut microbial communities were different between the CC group and the HCs group.

3.3 Differential gut microbiota in cervical cancer patients

Based on 16s rRNA sequencing analysis, we next used the MetagenomeSeq analysis to avoid the influence of the Rarefaction process on the accuracy of the results to further explore differences between the CC group and the HCs group. In the MetagenomeSeq analysis, we found that *Ruminococcus 1*, *Agathobacter*, *Ventriosum group*, *Ruminococcus 2*, and *Bacteroides* were all differential (p<0.01). Among them, *Ruminococcus 2* showed the most significant difference (p<0.001) (Figure 3A). We then used Leave-one-out cross-validation to build a random forest classification model (p<0.05). Random forest analysis and ROC of core species were performed in randomly generated decision tree fitting input samples, which first filtered species level data and selected core microorganisms for analysis (min relative = 0.001, min ratio = 0.7, p<0.05 for significant differences), and found significant differences for *Ruminococcus 2* and *Barnesiella* (Figure 3B). The effect of this classification model was assessed using the receiver operating characteristic (ROC) curve with the area under the ROC curve (AUC) as 0.92 (Figure 3C). It indicated that low *Ruminococcus 2* in the gut flora family is most closely related to cervical cancer. In addition, LEfSe also found that microbes were the major contributor to sample differences (Figure 3D, LAD score > 2, p < 0.05). However, in our LEfSe analysis, all differential species were more abundant in the HCs group. Specific species have been identified in our analysis, which could be further studied for their association with cervical cancer progression. Among them, *Ruminococcus 2* might be a potential marker in cervical cancer patients.

3.4 Altered functional composition of gut flora in patients with cervical cancer

To study the altered gut microbial function in CC patients, PICRUSt2 was used to annotate the OUT sequence and predict the function of 16S rRNA sequencing analysis. Through the combination of KEGG and other databases, gene annotation and biological interpretation were carried out. We detected the microbial functional abundance of the gut flora in the CC group and the HCs group, and the

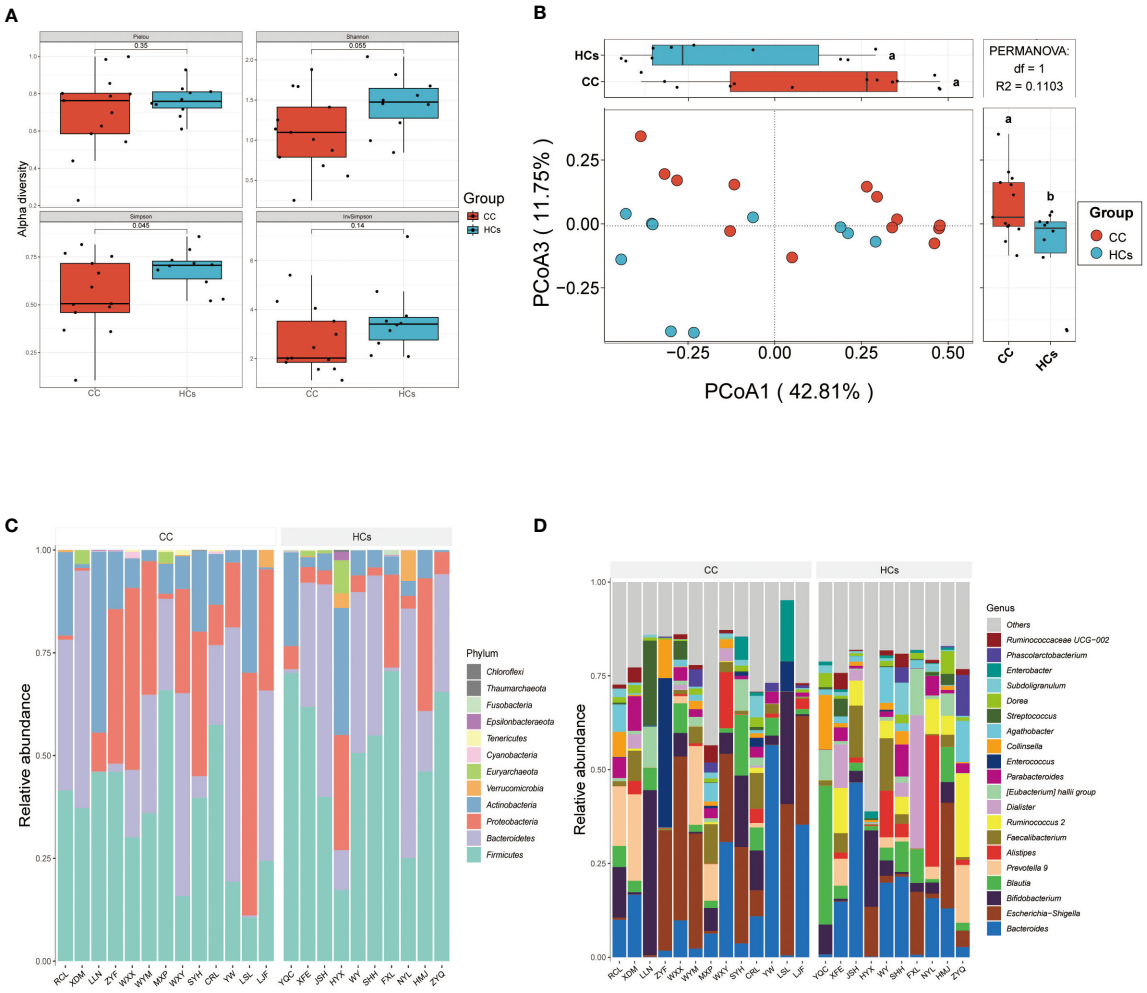

FIGURE 2
Gut microbial diversity differs in patients with cervical cancer compared to healthy controls. **(A)** α-diversity analysis (Pielou, Shannon, Simpson, and InvSimpson index). **(B)** PCoA analysis based on Pearson Distance and Bray-Curtis distance matrices. Each sphere represents one sample. Samples are separated into two groups. **(C)** The two groups' average compositions and relative abundance at the phylum level. **(D)** The two groups' average compositions and relative abundance at the genus level. aa $p>0.05$. ab $p<0.05$.

functions of the differentially expressed genes were mainly concentrated in REDOX reaction, biosynthesis of other secondary metabolites, amino acid transport, and metabolism ($p<0.05$, Figure 4A). To validate functional alterations in the gut microbiome, mNGS testing was applied for microbial function analysis. The fecal microbial samples from 9 participants closest to the test date (5 from the CC group and 4 from the HCs group) were selected for mNGS testing. We conducted the functional difference analysis of mNGS testing and found 41 significantly different results. The top 10 metabolic pathways with differential abundance were selected for distribution analysis (Figures 4B–K, and it was found that the abundance of 10 differential metabolic pathways was increased in the tumor group, including the superpathway of thiamine diphosphate biosynthesis III (THISYNARA-PWY), purine nucleotides degradation II (PWY-6353), fucose degradation (FUCCAT-PWY), superpathway of adenosylcobalamin salvage from cobinamide I (COBALSYN-PWY), methylerythritol phosphate pathway II (PWY-7560), thiamine phosphate formation from pyrithiamine and oxythiamine (PWY-7357), seleno-amino acid biosynthesis (PWY-6936), L-arginine biosynthesis I (ARGSYN-PWY), and superpathway of L-threonine biosynthesis (THRESYN-PWY). These results indicated significant differences in microbiota functions between the CC and HCs groups mainly falling in the production of precursor metabolites and energy, as well as in the biosynthesis of amino acids.

3.5 Relationship between gut flora and clinical indices

We also evaluated the correlation between clinical indices and gut flora. BMI, disease, and stage were found to be strongly correlated with the abundance of bacterial genera in the CC group (Figure 5A). BMI was negatively correlated with *Bacteroides Plebeius* ($p<0.05$). Disease and stage were significantly negatively correlated with *Ruminococcus 2* ($p<0.01$). And stage showed a positive correlation with *Escherichia Shigella* ($p<0.05$). In this study, distinct points were used to represent various samples while also including clinical markers and microbiological abundance for analysis. The length of

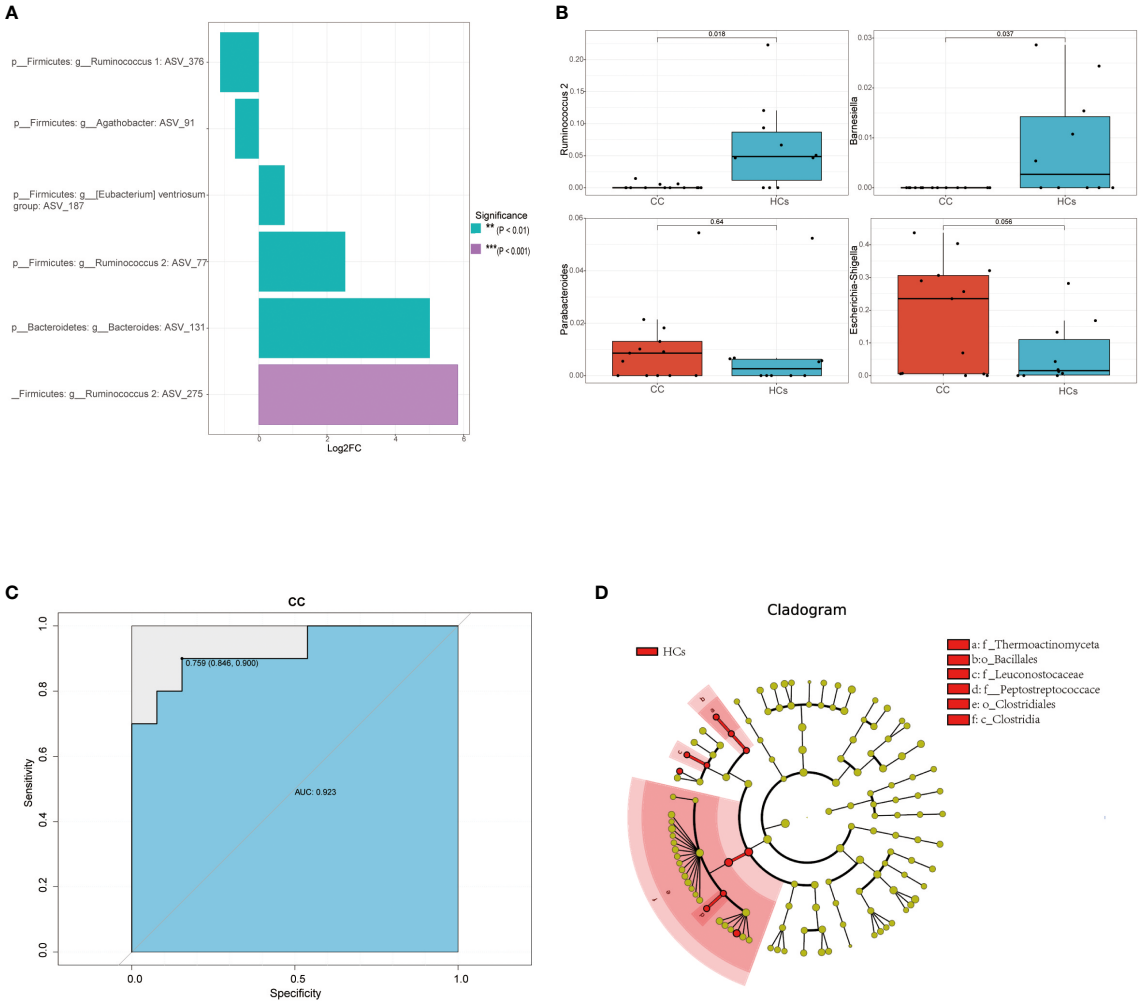

FIGURE 3
Differential gut microbiota in cervical cancer patients. **(A)** Linear discriminant analysis effect values species bar graph based on MetagenomeSeq analysis. **(B)** Core microorganisms based on random forest analysis (min_relative = 0.001, min_ratio = 0.7, p<0.05 for significant differences). **(C)** achieving an AUC value of 0.923 **(D)** Taxonomic cladogram from LEfSe, depicting the taxonomic association between the fecal microbiome communities from CC and HCs group. ***p*<0.01, ****p*<0.001. AUC, area under the curve; CC, cervical cancer; HCs, healthy controls.

the arrow, which indicated the intensity of each environmental factor's influence on community transformation, reflected the extent of the environmental factor's influence. The arrows from the origin represented various environmental factors. The correlation between the patient factor and the coordinate axis is indicated by the angle between the arrow and the axis, and the smaller the angle, the stronger the correlation. We calculated that Tumor-diameter (p=0.014) was associated with microbial abundance, but the magnitude of the correlation could not be determined (Figure 5B). These data further indicated that *Ruminococcus 2* was associated with cervical cancer disease and stage, making it a promising marker.

3.6 The mNGS testing of gut microbiota in patients with cervical cancer

In addition to the functional tests described above, we validated the 16S rRNA sequencing analysis results with mNGS testing. The results suggested no significant difference in α-diversity between the two groups (Figure 6A). The difference in β-diversity between the two groups was along the second axis of the Pearson and Bray-Curtis distances, explaining 18.7% of the total variation (Figure 6B). The abundance of *Bacteroides* was significantly increased at the genus level (Figure 6C). At the species level (Figure 6D), the abundance of *Firmicutes* and *Bacteroidetes* was significantly reduced. The above results were consistent with 16S rRNA sequencing analysis between CC and HCs groups.

4 Discussion

In recent years, many studies have shown that gut microbiome dysregulation is increasingly associated with the development of tumors near or far from the gut tract (Xue et al., 2018; Cheng et al., 2020; Yang et al., 2021). For example, in liver cancer (Yu and Schwabe, 2017; Ma et al., 2018; Zhang et al., 2021a) and breast

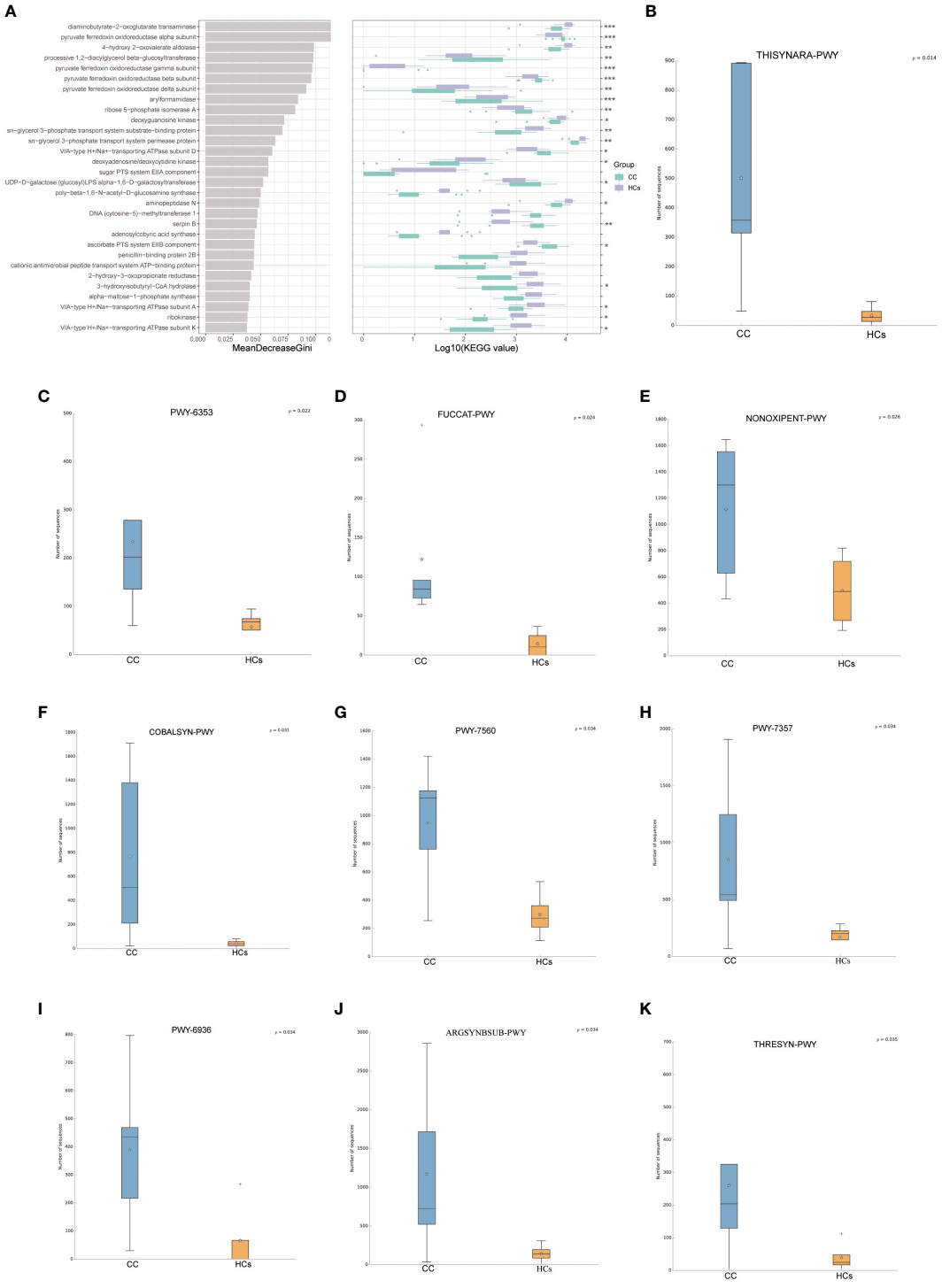

FIGURE 4
Predicted functionality of the fecal microbiota. **(A)** 30 enriched pathways with the most significant differences between the CC and HCs groups were identified based on KEGG by 16s rRNA sequencing analysis. **(B–K)** The top 10 metabolic pathways with differential abundance by mNGS testing.

cancer (Kwa et al., 2016; Chen et al., 2019), it has been confirmed that the transformation of primary bile acids into secondary bile acids by microorganisms may cause DNA damage, hepatotoxicity, change of NK T cell concentration and carcinogenesis. In breast cancer, gut bacteria lead to human steroid metabolism disorder, making estrogen content and distribution changes (Kwa et al., 2016; Xue et al., 2018). Thus, the investigation of gut microbiota associated with cancer progression gives much insight into related clinical applications. However, studies on the gut flora of cervical cancer are quite limited so far.

This study collected fecal samples from cervical cancer patients and normal controls to compare the different microbial signatures. In addition, the changes in gut microflora and their corresponding functions were also studied. Compared with the healthy control

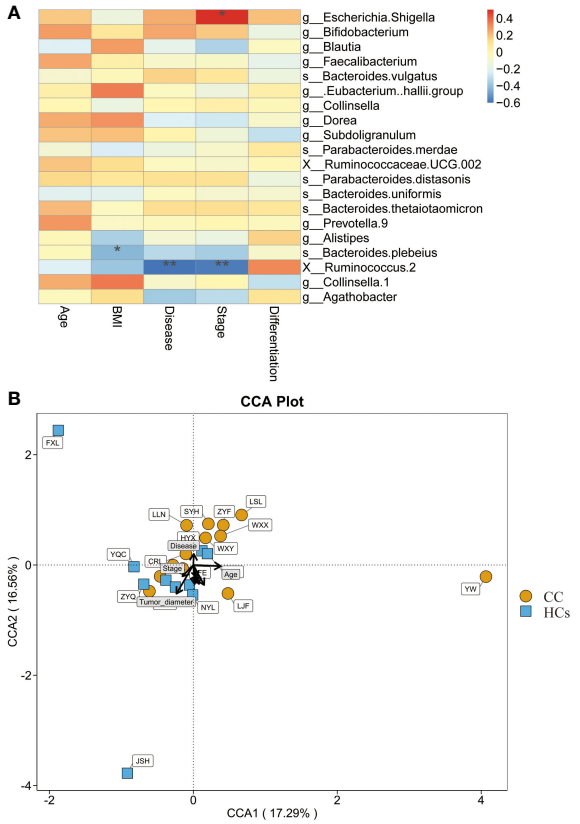

FIGURE 5
Relationship between gut flora and clinical indices. **(A)** Heatmap shows the correlation between clinical indices and gut flora. **(B)** CCA shows the correlation between clinical indices and microbial abundance. *p<0.05, **p<0.01. CCA, canonical correspondence analysis.

group, the results showed significant differences in gut microflora classification composition and diversity in patients with cervical cancer. Although α-diversity did not show a difference in the abundance of gut flora, β-diversity indicated the difference between the CC group and HCs group. The composition of the gut microbiota of CC and HCs was dominated by *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. However, their abundance was significantly different between CC and HCs groups. BMI was negatively correlated with *Bacteroides Plebeius*. Disease and stage were significantly and negatively correlated with *Ruminococcus 2*. And stage showed a positive correlation with *Escherichia* Shigella. Interestingly, we found that *Ruminococcus 2* was most closely related to cervical cancer in the gut flora family. These findings provide preliminary evidence that those with cervical cancer have distinctive gut flora.

Numerous research has supported the association between gut microbiota and non-digestive disorders (Kwa et al., 2016; Mohajeri et al., 2018; Ochoa-Repáraz et al., 2018; Peirce and Alviña, 2019). However, most studies are based on low-throughput methods or cultures of microbial flora, such as real-time fluorescence PCR and 16S rRNA amplification denaturing gradient gel electrophoresis. These findings only represent a small portion of the whole microbe. As a result, it is still very difficult to comprehend the general state of the gut microbial population and the accompanying microbial ecology and function. Since there is a lot of sequence variability among different bacteria, 16S rRNA genes of entire gut bacteria were chosen for sequencing in this work. These genes were typically grouped into 9 "hypervariable regions" (V1-V-9). Sequences that discriminate between a small number of distinct bacterial species or that identify particular bacterial species have been isolated through studies. In addition to 16S rRNA sequencing analysis, mNGS testing was added. The mNGS testing involves sequencing millions of DNA fragments in parallel (Behjati and Tarpey, 2013; Han et al., 2019). Piecing together individual reading fragments by mapping them to the reference genome. Each piece of DNA is sequenced multiple times, providing greater depth for accurate data and the discovery of different species. Therefore, this experiment has a better understanding than 16S rRNA sequencing analysis alone. In addition to diversity, the functions of some bacteria and their corresponding differential metabolic pathways were revealed.

In this study, the dominant expression of estrogen metabolism-related bacteria, such as *Bacteroides*, was also found in the dominant bacteria (Flint et al., 2012). Cervical cancer's onset and progression may be linked to estrogen metabolism-related bacteria. A previous study showed that estrogen helps cervical cancer using a mouse model (Elson et al., 2000). The transformation region of transgenic HPV16 mice was 5 times more sensitive to estrogen-induced squamous cell carcinogenesis compared to the rest of the reproductive tract. In addition, another study also reported higher cervical cancer analysis in women with the highest circulating estrogen levels (Chen et al., 2021; Wang et al., 2021). We also found that *Ruminococcus 2* was most closely related to cervical cancer in the gut flora family, which could be considered a potentially relevant biomarker for the prediction of cervical cancer development. It has been found that *Firmicutes*, especially *Ruminococcus*, played an important role in polysaccharide degradation (Setchell and Clerici, 2010). *Ruminococcus* plays an important role in human metabolism by converting cellulose into various nutrients of the host. *Ruminococcus* is also closely related to the intestinal barrier, cellular immunity, inflammation, and metabolism (Hall et al., 2017; Schluter et al., 2020; Rangarajan et al., 2022). A recent study found that the absolute abundance of *Ruminococcus 2* showed a positive correlation with the number of lymphocytes (Schluter et al., 2020). The human immune effect is enhanced when the abundance of Ruminococcus 2 is higher. However, whether *Ruminococcus 2* affects the occurrence and development of cervical cancer in the host requires further biofunctional studies (Goedert et al., 2015; van der Meulen et al., 2016).

The major shortcoming of this study is the number of specimens collected. Therefore, we cannot clearly distinguish whether different stages and precancerous lesions have an impact on the gut microflora. In addition to HPV, other risk factors, such as multiple sexual partners and different living environments, were not analyzed. At the same time, the correlation between different flora and metabolic pathways associated with the occurrence and development of cervical cancer cannot be clarified without further functional experiments. Further prospective studies of longitudinal sampling of the microbiota of patients with persistent HPV-positive precancerous lesions are needed to determine whether progressive disruption of the microbiota contributes to cervical cancer.

10.3389/fcimb.2023.1145950

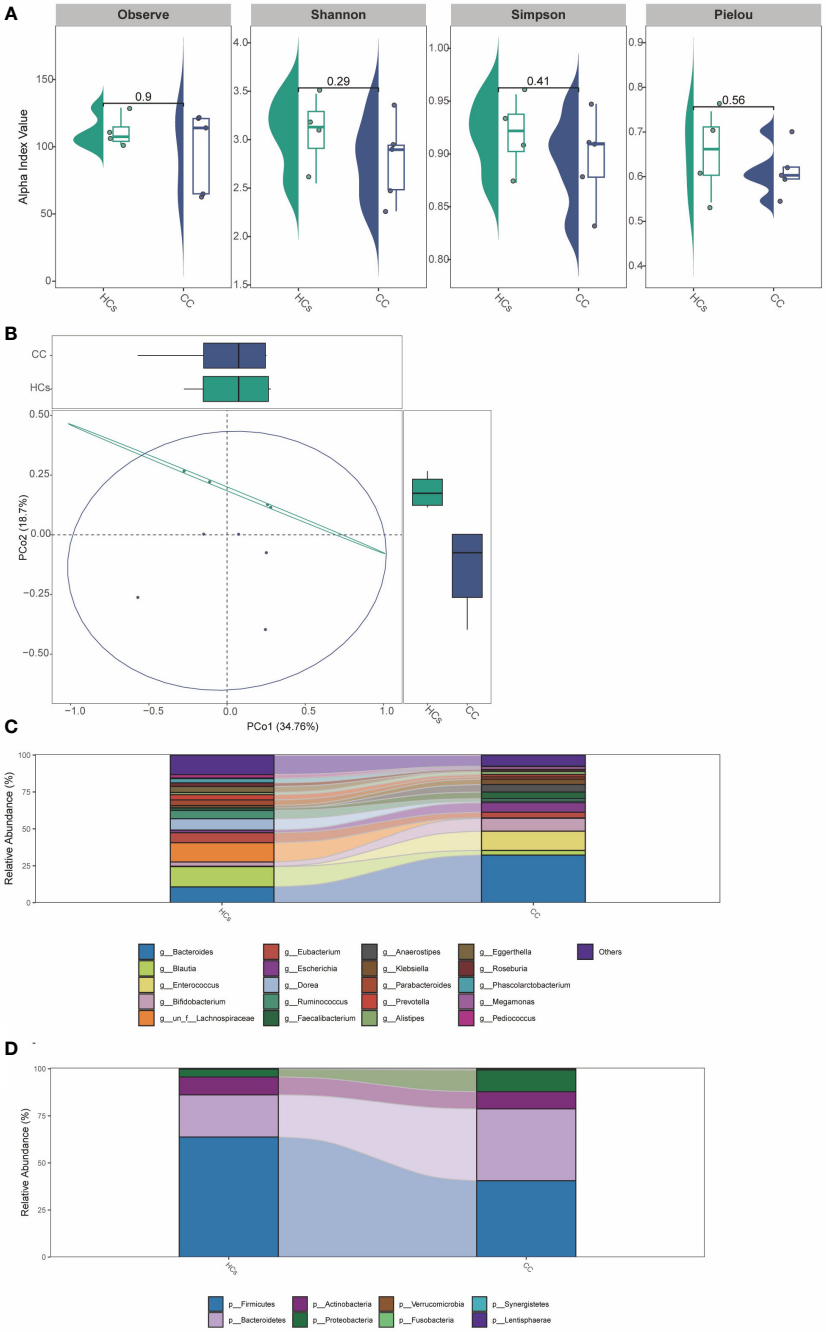

FIGURE 6
The mNGS testing of gut microbiota in patients with cervical cancer. **(A)** α-diversity analysis (Observe, Shannon, Simpson, and Pielou index). **(B)** PCoA analysis based on Pearson Distance and Bray-Curtis distance matrices. Each sphere represents one sample. Samples are separated into two groups. **(C)** The two groups' average compositions and relative abundance at the genus level. **(D)** The two groups' average compositions and relative abundance at the species level.

5 Conclusion

Cervical cancer patients have different gut microbiome diversity compared with healthy women. This difference is reflected both in terms of community structure, as defined by increased or decreased diversity of the gut microbiome, and in terms of functional composition. The presence of microbial composition and specific functional pathways that may be necessary for the development of cervical cancer have been compared using prospective data.

Data availability statement

The data presented in the study are deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) at

the National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences. The accession number is CRA009631.

Ethics statement

The studies involving human participants were reviewed and approved by the First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

Conceptualization: NL, RG, and LC. Funding acquisition: NL and LC. Project administration: LC. Supervision: LC. Validation: LQ, JZ, SD, MC, FW, and BQ. Visualization: LQ, JZ, SD, MC, and FW, and BQ. Writing-original draft: LC, LQ, and NL. Writing-review and editing: NL and LC. This work was carried out in collaboration among all authors. All authors contributed to the article and approved the submitted version.

Funding

Henan Provincial Medical Science and Technology Research Plan Joint Provincial and Ministry Youth Project (No.SB201902013), Scientific and Technological Project of Henan Province (No.182102310325 and 192102310069), Henan Province Colleges and Universities Innovative Talent Support Program (No.21HASTIT044), Henan Medical Education Research Project (No.Wjlx2020062), Henan Youth Talent Promotion Project (No.2020HYTP052), Central Plains Youth Top Talent Project, and High-level Talents Return to China for Research Funding Project.

Acknowledgments

We thank the participants for their contributions to this study.

Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1145950/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 16 March 2023
DOI 10.3389/fcimb.2023.1132472

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 27 December 2022
ACCEPTED 28 February 2023
PUBLISHED 16 March 2023

CITATION
Niu J, Wang J, Jia P, Zhang M and Wei E (2023) Clinical features and diagnostic value of metagenomic next -generation sequencing in five cases of non-HIV related *Pneumocystis jirovecii* pneumonia in children.
Front. Cell. Infect. Microbiol. 13:1132472.
doi: 10.3389/fcimb.2023.1132472

Clinical features and diagnostic value of metagenomic next -generation sequencing in five cases of non-HIV related *Pneumocystis jirovecii* pneumonia in children

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Background: *Pneumocystis jirovecii* (PJ) is an opportunistic pathogenic fungus, and PJ pneumonia (PJP) is a commonly problem in HIV-positive patients. While PJP is not caused by HIV, it generally advances rapidly and can quickly lead to severe respiratory failure. To improve pediatricians' understanding of the condition and aid early accurate diagnoses and therapy, we examined the clinical characteristics of five instances of non-HIV related PJP (NH-PJP) in children and the efficacy of metagenomic next-generation sequencing (mNGS) in its diagnosis.

Methods: From January 2020 to June 2022, five children with NH-PJP were admitted to the PICU of the First Affiliated Hospital of Zhengzhou University. We retrospectively summarize the clinical presentation, previous histories, routine laboratory findings, treatment, outcome of regression, and results of mNGS in these five children.

Results: Five male children between the ages of 11 months and 14 years had an acute onset on NH-PJP, three of the children had chest tightness after activity, shortness of breath and paroxysmal dry cough, — and two had high fever and dry cough. All five of the children had several flocculent high-density pictures in both lungs at the beginning of the disease, and lung auscultation revealed coarse breath sounds in both lungs, one of which was accompanied by a modest quantity of dry rales. PJ nuclear sequences were found in one patient and four patients' blood and alveolar lavage fluid. All five children were treated with Trimethoprim-sulfamethoxazole (TMP-SMX) in combination with Caspofungin and corresponding symptomatic treatment. Four patients were cured and one patient died.

Conclusion: Children commonly encounter an initial exposure to NH-PJP, which manifests as a high fever, dry cough, chest discomfort, dyspnea that worsens over time, fast disease progression, and a high death rate. The clinical presentation of children with PJ infection should be taken into consideration along with the results for diagnose. mNGS has higher sensitivity and a shorter detection period compared to identification of PJP.

KEYWORDS

clinical features, MNGs, diagnosis, infection, NH-PJP

1 Introduction

Pneumocystis jirovecii (PJ) is an opportunistic pathogenic fungus, and PJ pneumonia (PJP) is commonly found in HIV-positive patients. With the use of chemotherapy or immunosuppressive medication in recent decades, the prevalence of non-HIV related PJ (NH-PJP) has been rising annually (Catherinot et al., 2010; Avino et al., 2016). The clinical presentation of PJP varies greatly between HIV-infected and non-HIV-infected patients (Bienvenu et al., 2016; Liu et al., 2019). In the HIV- infected population, patients with PJP usually present with sub-acute onset of gradual dyspnea, nonproductive or minimally productive cough, low grade fever and malaise. Early in the course of infection patients may be asymptomatic (Oladele et al., 2018). NH-PJP progresses rapidly and is difficult to diagnose correctly, thus leading to severe respiratory failure (Cordonnier et al., 2016; Liu et al., 2019). NH-PJP mortality rates have been reported to range from 28% to 53%, which is significantly higher compared to HIV-infected patients (17% to 30%) (Cordonnier et al., 2016). NH-PJP symptoms include high fever, dry cough, chest pain, and progressively worsening dyspnea with oxygen and impairment. Patients with NH-PJP often present with symmetrical diffuse ground glass shadows in both lungs, along with patchy shadows, interstitial alterations, and air sac-like changes on computed tomography (CT) scan (McKinnell et al., 2012).

The prognosis for children with NH-PJP depends greatly on early and timely diagnosis, precise anti-PJP treatment, and awareness of the seriousness and rapid progression of NH-PJP (Lu et al., 2022). In recent years, a molecular biology technique called metagenomic next-generation sequencing (mNGS) has been developed, which has the potential to identify more than 15,000 pathogenic microorganisms with known genome sequences (Gu et al., 2019). However, there are few clinical examples of applying bronchoalveolar lavage fluid (BALF) mNGS (BALF-mNGS) to diagnose PJP. Herein, we have retrospectively analyzed the clinical data of five children with NH-PJP and the results of mNGS examination. To increase pediatricians' comprehension of the condition and aid assist, as well as to promote prompt and accurate treatment, we summarize and evaluate the clinical characteristics of NH-PJP in children and discuss the diagnostic utility of mNGS technology in confirming PJP.

2 Methods

2.1 Study design and participants

In this retrospective study, we consecutively enrolled PJP patients who were admitted to the PICU of the First Affiliated Hospital of Zhengzhou University, from January 2020 to June 2022. Patients were eligible for enrollment if they met all the following criteria: (1) immunocompromised conditions, including but not limited to hematologic malignancies, solid tumors, rheumatic diseases, long-term systemic use of corticosteroids (0.3 mg/kg/day of prednisone equivalent for > 3 weeks), use of immunosuppressive agents (including chemotherapeutic agents for malignancies, but not corticosteroids), solid organ transplantation and hematopoietic stem cell transplantation;(2) typical clinical manifestations of PJP, including fever, cough, dyspnea, and progressive hypoxemia; (3) radiologic findings suggestive of PJP in bilateral lungs that newly emerged on CT scans; (4) BALF and/or blood samples were collected for mNGS. Furthermore, we collected patients' baseline information, clinical features, laboratory and imaging examination results, diagnoses, treatments, and outcomes. Patients were excluded if they met any of the following criteria: (1) age≥18 years old; (2) HIV infection; (3) mNGS was not performed; (4) medical record was incomplete.

2.2 mNGS protocol

2.2.1 Sample processing and sequencing

First 3-4mL of blood was drawn from patients, placed in EDTA tubes, and stored at room temperature for 3–5 minutes before plasma separation and centrifuged at 1,600 g for 10 min at 4°C within 8 hours of collection. Plasma samples were transferred to sterile tubes. DNA was extracted from 300 μL of plasma using the TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH, Beijing, China) following the manufacturer's instructions. The extracted DNA specimens were used for the construction of DNA libraries.

BALF was collected based on the standard clinical procedure. Briefly, 20 mL saline was injected into a segmental bronchus and drawn back after a while. Next, 3 mL of BALF was inactivated at 65°C for 30 minutes immediately after collection. Subsequently, a 1.5-mL

microcentrifuge tube with 0.5-mL sample and 1 g 0.5-mm glass beads were attached to a horizontal platform on a vortex mixer and agitated vigorously at 2800–3200 prm for 30 min. Finally 0.3-mL sample was collected into a new 1.5-mL microcentrifuge tube and DNA was extracted using the TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH) according to the manufacturer's instructions.

According to the protocol of the BGISEQ-50 sequencing platform, the DNA library was constructed through DNA fragmentation, end-repair, adapter-ligation, and PJR amplification. The constructed library was qualified by Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA) and Qubit 2.0. The qualified double-strand DNA library was transformed into a single-stranded circular DNA library through DNA-denaturation and circularization. DNA nanoballs (DNBs) were generated from single-stranded circular DNA using rolling circle amplification (RCA). The DNBs were qualified using Qubit 2.0. Qualified DNBs were loaded into the flow cell and sequenced (50 bp, single-end) on the BGISEQ-50 platform.

2.2.2 Bioinformatic analysis

High-quality sequencing data were generated by removing low-quality and short (length < 35 bp) reads using in-house software, followed by computational subtraction of human host sequences mapped to the human reference genome (hg19) using Burrows–Wheeler Alignment. After the removal of low-complexity reads, the remaining data were classified by simultaneously aligning to four Microbial Genome Databases, consisting of viruses, bacteria, fungi, and parasites. The four Microbial Genome Databases were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/). RefSeq contains 4,061 whole-genome sequences of viral taxa, 2,473 bacterial genomes or scaffolds, 199 fungi, and 135 parasites associated with human diseases. The number of unique alignment reads was calculated and standardized to get the number of reads stringently mapped to pathogen species (SDSMRN) and the number of reads stringently mapped to pathogen genus (SDSMRNG).

3 Results

3.1 General information

In this study, five children were eventually enrolled (Figure 1).

Case 1, male, 3 years old, with underlying disease of autoimmune encephalitis, was treated with rituximab (375 mg/m^2, two doses) and regular maintenance therapy with prednisone (10 mg/d) during the treatment of the underlying disease.

Case 2, male, 11 months, with acute lymphoblastic leukemia as the underlying disease, was treated with the PVDL regimen (Prednisone, Vincristine, Daunorubicin and L-Asparaginase) at presentation.

Case 3, male, 8 years old, was in a post-transplant state and was taking tacrolimus regularly.

Case 4, male, 14 years old, in post-renal transplant status, on regular Tacrolimus.

Case 5, male, 6 years old, with underlying disease of special sarcoma, treated with VDC regimen (Vincristine, Cyclophosphamide and Doxorubicin) before the onset of the disease. five children had no abnormal personal, growth and developmental, or family history.

3.2 Clinical manifestations and signs

Cases 1, 4, and 5 started with chest tightness after activity, shortness of breath, and paroxysmal dry cough, and developed fever with a fever peak of 39°C or higher on the 2nd_4th day of illness. Cases 2 and 3 started with fever peaking at 39°C with dry cough. Five children developed respiratory distress within 3_4 days after the onset of the disease and were mechanically assisted with ventilation on day 3 in case 1, on day 10 in case 2 and on day 7 in cases 3, 4 and 5. In case 2, coarse breath sounds and a few dry rales could be heard in both lungs at the onset of the disease, and in the other four children, coarse breath sounds could be heard in both lungs as far as possible.

3.3 Laboratory test results

Peak LDH (233_2002) U/L, peak BDG (10.00_2470.6) pg/mL, and CD4 lymphocyte count (18.32_985)/μL in five children (Table 1).

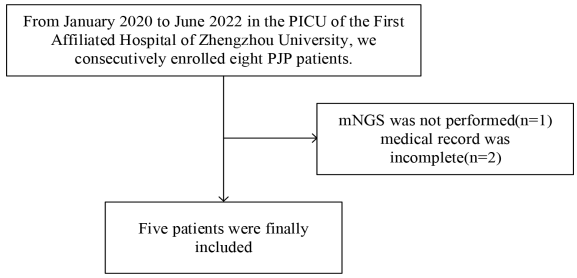

FIGURE 1
Flow diagram of the patients included in the study.

TABLE 1 The results of CRP, PCT, LDH, BDG and CD4 lymphocyte count in five children.

NO.	peak of CRP (0-5mg/L)	peak of PCT (0-0.2ng/L)	peak of LDH (200-600U/L)	peak of BDG (0-95pg/mL)	CD4 lymphocyte count (550-1440/uL)
Case1	42.95	2.03	2002	584.32	985
Case2	90.23	5.84	233	<10	NA
Case3	35.66	0.864	174	100.37	840
Case4	101	4.83	502	254.28	282.26
Case5	51.34	0.237	834	2470.6	18.32

NA, Not Answer.

3.4 Chest imaging results

All five children showed multiple flocculent high-density images in both lungs on chest CT (Figure 2).

3.5 mMGS and pathology test results

3.5.1 mNGS test results

The five children had gene sequence numbers of 340, 3, 1797, 27025, and 63156, respectively, with mNGS showing positive PJP. These children were co-infected with other pathogens, such as Streptococcus pneumoniae and cytomegalovirus (Table 2).

3.5.2 Regie's stain results

Case 1 and case 4 results suggest positive, case 3 and case 5 results suggest negative, case 2 was not tested.

PJ and encapsulation were seen in the BALF of case 1 (Figures 3A, B) and case 4 (Figures 3C, D).

3.5.3 Fungal immunofluorescence staining results

No fungus was seen under fungal immunofluorescence staining in all four children (case 2 was not tested).

3.6 Treatment history and regression

Cases 1, 3, 4 and 5 were transferred to the PICU of the First Affiliated Hospital of Zhengzhou University within 3 to 4 days after the onset of illness without targeted treatment, and were treated immediately with TMP-SMX combined with caspofungin anti-infective therapy, alveolar lavage and mechanical assisted ventilation support. Among them, cases 1, 3 and 4 were treated with methylprednisolone, and cases 1 and 4 were treated with intravenous immunoglobulin. All four children recovered. In case 2, blood mNGS was checked on the 3rd day after fever, which tested positive for *Enterococcus faecalis* and *Streptococcus vestibularis*. After 9 days of combined treatment with meropenem and teicoplanin and 3 days of caspofungin treatment, the child still had an intermittent fever and respiratory distress, and the oxygen saturation was maintained at about 90% by transdermal oxygen measurement under 5 L/min of face mask inhalation. Case 1, 3, 4 and 5 were cured and Case 2 was dead (Table 3).

4 Discussion

In vitro cultivation of unusual fungus, PJ, is extremely challenging. Traditionally, diagnosis relies on the presence of

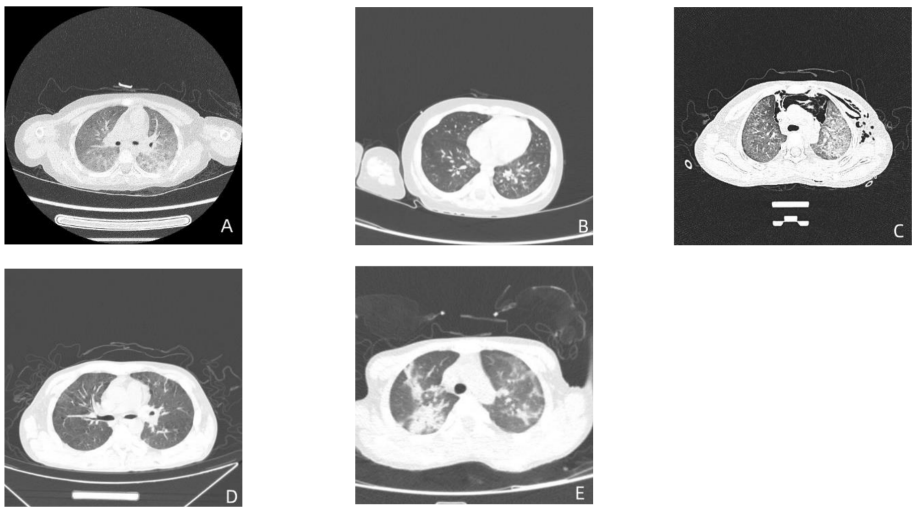

FIGURE 2
Pulmonary CT results in five children. The figure **(A–E)** are the CT images of the lungs of case 1-5 in order.

10.3389/fcimb.2023.1132472

TABLE 2 Results of mNGS in five children.

NO.	Sample of mNGS	Number of gene sequences	Relative abundance	Combination of other pathogens
Case 1	BALF	340	69.11%	*S. pneumoniae* Cytomegalovirus Herpes simplex virus
Case 2	Blood	3	100%	*S. pneumoniae*
Case 3	BALF	1797	89.19%	*S. pneumoniae* Cytomegalovirus
Case 4	BALF	27025	3.16%	Deficient oxygen-depleted bacteria
Case 5	BALF	163156	96.79%	Klebsiella pneumoniae Acinetobacter baumannii Haemophilus influenzae

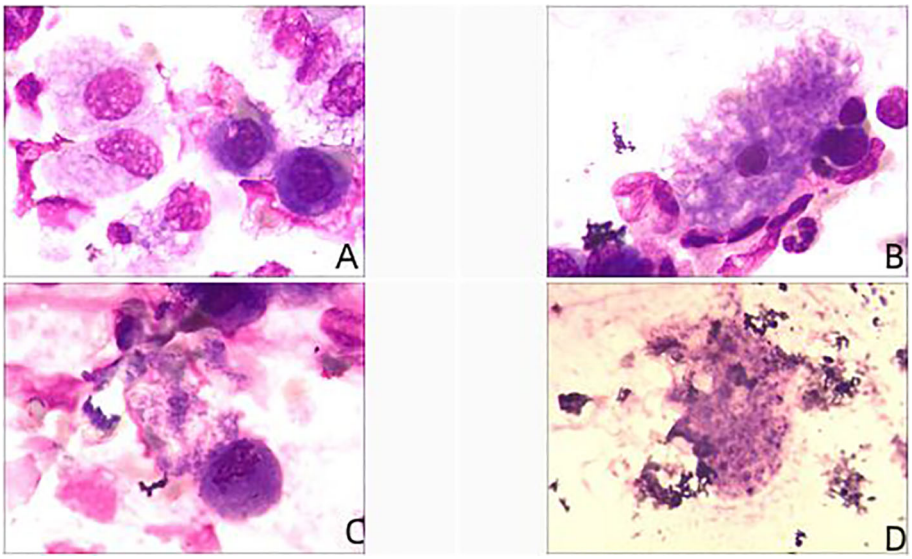

FIGURE 3
Regie's stain results of case 1 **(A, B)** and case 4 **(C, D)**.

TABLE 3 Treatment and outcome of five children.

NO.	Time of starting treatment	Time of drug treatment	Improvement time after medication	Outcome
Case1	3	19	12	Cured
Case2	11	1	NA	Dead
Case 3	4	20	13	Cured
Case 4	4	20	13	Cured
Case 5	3	21	14	Cured

NA, Not Answer.

specific encapsulated or trophoblast cells in specimens such as BALF or sputum, radiological manifestations, and clinical symptoms (Bateman et al., 2020). Despite being simple and inexpensive, traditional microscopic examination is less sensitive for PJP because of the low fungal load. Despite having 100% specificity for the diagnosis of PJP, immunofluorescence and cytological staining only exhibited a 74% and 50% sensitivity, respectively, according to a meta-analysis (Senécal et al., 2022). In this study, PJ was detected among alveolar lavage fluid in only two children due to the limited sensitivity of the conventional assay.

Because of the significant limits of the conventional approach, a more efficient and sensitive assay is required to fulfill clinical demands.

PJP-induced lung inflammation and damage can result in an aberrant rise in LDH (Oladele et al., 2018). LDH has been confirmed to be a reliable non-specific marker for PJP, with a sensitivity of 96% and a specificity of 77% when LDH >379 U/L (Sun et al., 2021). Due to the limited sample size in this study, only three children had LDH levels over 379 U/L, which meant that we were unable to clearly establish the significance of LDH in the diagnosis of NH-PJP. Moreover, further large-scale studies are required to confirm whether LDH can indicate the severity of PJP disease and the degree of lung inflammation.

DG is a polysaccharide synthesized from D-glucan, which is a specific component of the fungal cell wall, accounting for more than 50% of the fungal cell wall composition. DG testing can be used as a diagnostic aid in patients at high risk for invasive fungal disease, especially in patients with blood disorders (Lu et al., 2011a; Lu et al., 2011b). It has a specificity of 75% and a sensitivity of 90% for the diagnosis of PJP (Del et al., 2020).

In this study, case 2 was tested for BDG level only upon disease onset; he was not examined again throughout the consultation. Further, BDG levels were checked many times throughout the consultation in the other four instances. In cases 1, 4, and 5, the BDG levels were significantly elevated, further suggesting the possibility of PJP infection. According to the European Conference on Infections in Leukemia (ECIL), a negative serum BDG is sufficient to exclude PJP; however, a positive serum BDG is not specific for the diagnosis of PJP, necessitating further tests to confirm the diagnosis (Maschmeyer et al., 2016).

In addition, despite the great sensitivity of the BDG test, it can still give false positive results in cases of hemodialysis, gram-negative bacteremia, severe mucositis or intravenous immunoglobulin and certain antibiotics. Therefore, the BDG level alone cannot be used to diagnose PJP (Li et al., 2015). Clinical manifestations and additional laboratory test results must be paired with the PJP diagnosis in order to confirm it (Mercier et al., 2019).

Polymerase chain reaction (PCR) for PJ was initially developed in the 1980s with primers against the pneumocystis mitochondrial large-subunit ribosomal gene (Dunbar et al., 2020). PCR has been shown to be more sensitive for detection of PJP compared to staining methods in patients with and without HIV (Lu et al., 2011a; Lu et al., 2011b). However, mNGS outperformed the conventional method (i.e., *in vitro* culture, PCR) in the detection of MTB, bacteria, fungi, mycoplasma, and viruses (Huang et al., 2021).

The advantages in mNGS over traditional detection methods include a shorter detection cycle, a wider detection range, and a higher positive rate. This makes mNGS particularly suitable for conditions such as pathogenic bacteria that cannot be clarified by traditional detection methods or for clinical aspects that require rapid clarification of pathogenic bacteria. In comparison with BDG and hexamine silver staining, mNGS showed a sensitivity of 100% and a specificity of 96.3% in the diagnosis of PJP, according to research by Jiang et al. (2021). Liu et al. assessed the effectiveness of serum BDG and BALF-mNGS in aiding the diagnosis of PJP in their study (Liu et al., 2021). BDG >88.6 pg/mL and mNGS sequence number >14 exhibited 90% sensitivity and 14% specificity in diagnosing PJP. mNGS can assist in the diagnosis of PJP more efficiently and accurately than infectious immunofluorescent staining (Liu et al., 2021; Lu et al., 2022).

However, case 2 tested negative when the blood sample was tested on day 5 after the onset of the disease and only tested positive when the blood sample was tested again for mNGS on day 10 after disease onset. In four of the five children in our study, PJ was measured when BALF was tested within 3_5 days after the onset of the disease. This phenomenon suggests that BALF should be utilized as the test sample as much as possible in the clinical use of mNGS for the diagnosis of PJP. If only the blood specimen can be tested and the clinical manifestations and laboratory findings are highly suggestive of PJP, PJP cannot be excluded because of the first negative result, and mNGS should be performed at least once, but ideally more times. In addition, it is important to keep in mind that the microbial sequences reported by mNGS include potentially pathogenic organisms. As a result, the clinical setting must be properly considered when interpreting mNGS data. Although the list can help to qualify the range of pathogenic organisms, mNGS cannot yet accurately determine whether the flora is colonized or infected.

PJ was detected in all five patients using mNGS with a sensitivity of 100%, whereas when PJ and encapsulation were discovered using pathological staining and microscopy on tissues from four patients, the sensitivity was only 50%. The mNGS experimental cycle is shorter than the pathology assay, with data available in 48 hours as opposed to the pathology assay cycle, which is typically 3 days, and the mNGS assay was more sensitive in this study. The pathology results are more influenced by the technician's experience, while it is not necessary for the technician to be familiar with PJ for mNGS to simultaneously identify and compare all microbial genomes in a sample for analysis. The diagnosis PJ infections can be greatly aided using mNGS, which can also help doctors to quickly identify pathogenic organisms and administer prompt therapy to enhance the effectiveness of their interventions.

Several limitations of the study should be mentioned. First, the major limitation of this study is the small number of patients, due to the rarity of the disease. Second, this is a single center retrospective study; thus, intrinsic bias was unavoidable. Third, diagnostic performances of mNGS and PCR were not compared in this study because PCR of PJ was not routinely performed in our hospital. This line of query should be explored in future work.

In conclusion, to lessen the burden of NH-PJP patients, there is an urgent need for improved preventive and treatment measures. This is supported by the epidemiology, morbidity, and mortality of NH-PJP. Patients with suspected PJP should start anti-PJP specific medication immediately to prevent treatment delays brought on by diagnostic procedures such as bronchoalveolar lavage, which increases the need for mechanical assisted ventilation and disease mortality. The most crucial element in reducing NH-PJP related mortality is a straightforward, precise, and easily accessible diagnostic approach. The combination of BALF-mNGS should be

used clinically whenever possible to improve the detection rate of NH-PJP, but the results should be interpreted with a comprehensive consideration of the actual clinical situation because mNGS is a new microbiological assay with some advantages over traditional assays in identifying Pneumocystis.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ebi.ac.uk/ena/browser/view/PRJEB59277.

Ethics statement

The studies involving human participants were reviewed and approved by The First Affiliated Hospital of Zhengzhou University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

JN and JW collected the original clinical data and processed the statistical data. JN drafted and edited the manuscript. PJ and MZ participated in the design, and EW revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Henan Province Higher Education Key Research Project Plan (grant no. 21A320071).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at:https://www.frontiersin.org/articles/10.3389/fcimb.2023.1132472/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 27 March 2023
DOI 10.3389/fcimb.2023.1131258

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 24 December 2022
ACCEPTED 30 January 2023
PUBLISHED 27 March 2023

CITATION
Zhao X, Duan M-X, Lu Y-Y, Bai L-P and Zhao X-Y (2023) Short-term prognostic analysis of patients with systemic lupus erythematosus co-infection and comparison of mNGS and conventional microbiological test results.
Front. Cell. Infect. Microbiol. 13:1131258.
doi: 10.3389/fcimb.2023.1131258

Short-term prognostic analysis of patients with systemic lupus erythematosus co-infection and comparison of mNGS and conventional microbiological test results

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Objectives: Infection is one of the major causes of morbidity and mortality in patients with systemic lupus erythematosus (SLE), and as a new diagnostic technique, metagenomic next-generation sequencing (mNGS) is increasingly used for the pathogenetic detection of co-infected SLE patients. However, conventional microbiological testing (CMT) is still the gold standard for pathogenic diagnosis, and the specific diagnostic efficacy of mNGS versus CMT in such patients is not known. In addition, there are few studies on the short-term prognosis of co-infected SLE patients.

Methods: This study retrospectively included 58 SLE patients with co-infection admitted to the First Affiliated Hospital of Zhengzhou University from October 2020 to August 2022. Patients were divided into a survivors (n=27) and a non-survivors (n=31) according to their discharge status. Baseline characteristics and etiological data were collected and statistically analyzed for all patients during their hospitalization. The sequential organ failure assessment (SOFA) score, acute physiology and chronic health evaluation (APACHE) II and systemic lupus erythematosus disease activity index (SLEDAI) were calculated for each patient to assess the predictive ability of the 3 scores on the short-term prognosis of SLE patients. The mNGS and CMT culture results were also compared to clarify the flora characteristics of patients with SLE infection.

Results: More patients in the non-survivors had renal impairment, neurological manifestations, multiplasmatic cavity effusion and gastrointestinal manifestations compared to the survivors ($p < 0.05$). The SOFA score, APACHE II and SLEDAI were significantly higher in the non-survivors than in the survivors ($p < 0.01$). There were also significant differences between the two groups in several tests such as hemoglobin, platelets, albumin, total bilirubin, C-reactive protein (CRP), procalcitonin (PCT), and complement C3 ($p < 0.05$). In addition, the absolute values of T lymphocytes, CD4+ T cells and CD8+ T cells were smaller in the non-survivors than in the survivors ($p < 0.05$). The most common type of infection in this study was pulmonary infection, followed by bloodstream infection. mNGS and

CMT positivity rates were not significantly different among patients in the non-survivors, but were significantly different among patients in the survivors (p=0.029). In-hospital survival of patients with SLE infection could be predicted based on the SOFA score in relation to 6. For patients with SOFA <6, we recommend earlier mNGS testing to identify the pathogen and improve patient prognosis.

Conclusions: For SLE patients with co-infection, in-hospital survival can be predicted based on SOFA score. For patients with SOFA <6, advising them to complete mNGS testing as early as possible may improve the prognosis to some extent.

KEYWORDS

metagenomic next-generation sequencing, conventional microbiological testing, systemic lupus erythematosus, infection, short-term prognosis

1 Introduction

Systemic lupus erythematosus (SLE) is a difficult-to-evaluate multisystem autoimmune disease most commonly seen in women of childbearing age, with a prevalence ratio of approximately 9:1 between men and women (Smith and Gordon, 2010; Lisnevskaia et al., 2014). The clinical manifestations of the disease are diverse and can involve multiple systems and sites such as the cardiovascular system, central nervous system, kidneys, lungs, and eyes. Its course is mostly insidious, slow and recurrent, or it may deteriorate suddenly and even lead to acute progression or rapid death (Esposito et al., 2014). The etiology of the disease is complex and is associated with multiple mechanisms of genetic, environmental, innate and adaptive immunity (Wahren-Herlenius and Dörner, 2013). The life expectancy of SLE patients has improved considerably in the last two decades, but the mortality rate is still high, about three times higher than that of the general population (Singh and Singh, 2020). Several studies have pointed out that infections are highly associated with morbidity, hospitalization, and mortality in SLE patients and that SLE patients have a wide range of infections, with both bacterial and viral infections being common and having typical or atypical clinical manifestations. There are fewer studies on SLE patients with co-infections.

The Sequential Organ Failure Assessment (SOFA) score is commonly used to measure the severity of organ system dysfunction and failure and is primarily used to assess the acute morbidity of critical illnesses (Lambden et al., 2019; Pölkki et al., 2022). Acute physiology and chronic health evaluation (APACHE) II was developed in 1985 for critically ill patients in all disease categories in the ICU and can assess chronic health status (Sadaka et al., 2017). Systemic lupus erythematosus disease activity index (SLEDAI) is a score recommended by the 2020 Chinese SLE guidelines and is a predictor of hospitalization for infection (Petri and Genovese, 1992). In this study, the above three scores were included to investigate whether they differed between patients in the survivors and non-survivors and to explore their predictive ability for in-hospital survival of patients with SLE infection.

The metagenomic next-generation sequencing (mNGS) assay is a novel technology that has made a landmark contribution to the etiologic diagnosis of infectious diseases. Its high sensitivity allows it to detect a broader spectrum of pathogens than conventional microbiological testing (CMT) (Chen et al., 2021). However, CMT is still the gold standard for pathogenic diagnosis, and both have advantages and disadvantages in the etiologic diagnosis of infection. mNGS and CMT have unclear diagnostic positivity rates and diagnostic efficacy in SLE patients. We will investigate the characteristics of microbiota distribution in SLE patients and compare the diagnostic efficacy of mNGS with that of CMT to determine a more appropriate diagnostic strategy for early etiologic diagnosis, accurate use of antibiotics, and improved patient prognosis.

2 Methods

2.1 Study design and population

Co-infected SLE patients admitted by the First Affiliated Hospital of Zhengzhou University from October 2020 to August 2022 were retrospectively included in this study. Our inclusion criteria were: patients with a diagnosis made by a clinician and confirmed to be clinically infected after our retrospective review of each patient's medical records, which included clinical information, imaging examinations, and microbiological test results. The type of infection was determined by referring to the Centers for Disease Control (CDC)/National Healthcare Safety Network (NHSN) Definition of Surveillance for Specific Types of Infections (2019) and Internal Medicine Practice (15th edition) (Chen Haozhu et al., 2017; Centers for Disease Control and Prevention, 2019). Exclusion criteria: 1) not meeting the criteria for "infection" as defined in this study; 2) no mNGS test or no record; 3) age <18 years old; 4) pregnant women.

This study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (approval number: 2020-KY-429). The medical research involving human subjects in this study is in accordance with the ethical principles set forth in the Declaration of Helsinki (2013).

2.2 Data collection

Patients were screened according to our inclusion and exclusion criteria, and then the final inclusion was further divided into survivors and non-survivors based on discharge status. We identified and collected clinical data and examination test information from the case system during the patients' hospitalization, including gender, age, body mass index (BMI), history of smoking, history of drinking, duration of SLE, comorbidities (hypertension, type 2 diabetes, dyslipidemia, other autoimmune diseases), clinical manifestations of SLE, medication history, maximum body temperature, SOFA score, APACHE II, SLEDAI, whether tracheal intubation, tracheotomy, continuous renal replacement therapy (CRRT) and prophylactic antibiotics, ventilator use time, intensive care unit (ICU) time, hospital stay, and days of antibiotic use. To maintain baseline consistency, the laboratory values we recorded were all within 48h of the mNGS test, which included white blood cell count, hemoglobin, platelet count, glucose, potassium, creatinine, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), albumin, globulin, total bilirubin, ΔPH (absolute value of the difference between PH and 7.40), lactate, prothrombin time (PT), activated partial thromboplastin time (APTT), D-dimer, lactate dehydrogenase (LDH), creatine kinase isoenzyme (CKMB), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), procalcitonin (PCT), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), interferon-γ (IFN-γ), Complement C3, immunoglobulin G (IgG). In addition, the SOFA score, APACHE II, and SLEDAI in this study were all the highest values during the patient's current hospitalisation, and they represent the severity of the condition.

We also counted immunocytological indices of SLE patients, including the percentage and absolute values of T lymphocytes, B lymphocytes and NK lymphocytes. In addition, we collected the results of mNGS tests and CMT from the enrolled patients. Most patients in this study had more than one and more than one type of CMT test, and we recorded CMT results at or after the mNGS test and combined all CMT results.

2.3 mNGS method and process

This study was based on the NextSeq 550Dx platform (illumina, USA) for nucleic acid detection and sequencing. Patients with SLE were enrolled, and either infection site samples or peripheral blood samples (5 mL) were collected according to defined criteria. The infection site samples we collected included pericardial effusion, bronchoalveolar lavage fluid (BALF), cerebrospinal fluid, pus, and lung tissue. About 5mL of blood is collected using an anticoagulant collection container and stored and transported at room temperature. BALF, cerebrospinal fluid, pus and lung tissue need to be cryopreserved in dry sterile tubes. After proper storage and transport, samples were inactivated in a 56°C water bath for 30 minutes prior to nucleic acid extraction to reduce sample infectivity (Kampf et al., 2020; Pastorino et al., 2020; Woldesemayat et al., 2022). DNA was extracted by TIANamp Micro DNA kit after different pretreatments for different types of samples according to the manufacturer's instructions. DNA libraries were constructed by DNA fragmentation, end-repair, adapter ligation, and PCR amplification, followed by sequencing.

Agilent 2100 bioanalyzer (Agilent, USA) and ABI StepOnePlus Realtime PCR System were used for quality control of DNA library, including internal, negative and positive controls. The internal reference is from Arabidopsis thaliana and is provided by the sequencing manufacturer to enable tracking of the entire work process and control of process quality. And 15-20 samples containing negative controls are loaded in each macrogenomics sequencing batch and are used to detect environmental, reagent, and cross-sample contamination. Positive controls are real clinical samples proven to contain known pathogens (Lu et al., 2022). High-quality sequencing data was generated by removing low-quality and short reads (<35 bp in length), and then yield reads strictly aligned to pathogen species (SDSMRN) and reads strictly aligned to pathogen genus (SDSMRNG). The list of microorganisms obtained through the above analysis process was compared with an internal background database containing microorganisms present in more than 50% of samples in the laboratory over the past three months. Suspected background microorganisms were removed. Microorganisms with SDSMRN>50 and at least 3 times higher than the control group were considered as suspected pathogens, while the SDSMRN of suspected pathogens with SDSMRN <50 should be at least 5 times higher than the control group.

2.4 Study outcomes and definitions

The primary outcome in this study was in-hospital death, and the secondary outcome was the type of various infections.

CMT in this study included 1) blood culture 2) microbial staining and culture 3) sputum smear 4) serological tests for Epstein-Barr virus (EBV), cytomegalovirus (CMV) 5) CMV and EBV DNA testing 6) serum (Smith and Gordon, 2010; Esposito et al., 2014)-β-D-glucan test (G test) 7) serum galactomannan test (GM test) 8) T-SPOT tuberculosis (TB) test.

2.5 Statistics and analysis

The Shapiro-wilk test was used to verify that the continuous variables were normally distributed. Comparative analyses were performed using Chi-square tests for categorical variables, Fisher's exact test (when the expected value in a cell was at least <5), t-tests for two independent samples, and one-way analysis of variance (ANOV A) for continuous variables. The SOFA score, APACHE II, and SLEDAI were evaluated for their ability to discriminate the short-term prognosis of SLE patients, and the receiver operating characteristic (ROC) curves of the three scores were compared to calculate the area under the curve (AUC) and the best cutoff value. All statistical analyses were performed using IBM SPSS Statistics 27.0 (IBM, Armonk, NY, United States) and GraphPad Prism v9.0 (GraphPad Software, La Jolla, CA, USA). $P<0.05$ indicates that the differences were statistically significant.

3 Results

3.1 Results of recruitment

Figure 1 shows the screening process and clinical grouping of SLE patients. From October 15, 2020, to August 23, 2022, we reviewed 3752 SLE cases from the departments of respiratory and critical care medicine, ICU, rheumatology and infection at the First Affiliated Hospital of Zhengzhou University. A total of 3694 patients were excluded according to our inclusion/exclusion criteria, and 58 SLE patients with co-infection were included in the analysis. Based on the discharge of these patients, we divided them into a survivors (n=27) and non- survivors (n=31).

3.2 Clinical characteristics

Table 1 shows the clinical characteristics of the patients recruited in this study. Of the 58 patients with SLE, 12 were male and 46 were female. The mean age was 43.6 ± 15.6 years. By analysis, we found that there were more non-survivors with hypertension (15 (48.4%) vs. 5 (18.5%), p=0.017) but no patients with combined diabetes (0 (0.0%) vs. 6 (22.2%), p=0.006) compared to the survivors group. In terms of clinical manifestations of SLE, more people in the non-survivor group had renal impairment (24 (77.4%) vs. 10 (37.0%), p=0.002), neurological manifestations (20 (64.5%) vs. 5 (18.5%), p<0.001), multiplasmatic cavity effusion (19 (61.3%) vs. 8 (29.6%), p=0.016) and gastrointestinal manifestations (27 (87.1%) vs. 12 (44.4%), p<0.001), and more people had ≥5 simultaneous systemic manifestations (22 (71.0%) vs. 4 (14.8%), p<0.001) compared to the survivor group. In terms of laboratory values, the non-survivors had lower hemoglobin (86.8 ± 20.5 vs. 98.1 ± 16.3, p=0.026), platelets (93.0 ± 64.0 vs. 181.3 ± 82.1, p<0.001), albumin (24.7 (22.1-28.6) vs. 31.9 (27.9-34.5), p<0.001), and complement C3 (0.5 ± 0.3 vs. 0.8 ± 0.4, p=0.002), while glucose (7.8 (6.0-11.0) vs. 5.2 (3.8-7.0), p<0.001), total bilirubin (12.5 (7.3-24.5) vs. 5.8 (3.9-8.9), p<0.001), ΔPH (0.1 ± 0.0 vs. 0.0 ± 0.0, p=0.009), lactate (1.7 (1.2-2.8) vs. 1.1 (0.7-1.4), p=0.006), D-dimer (3.3 (0.8-4.8) vs. 1.0 (0.3-1.9), p<0.001), LDH (581.0 (352.0-791.0) vs. 372.0 (231.0-460.0), p=0.003), CRP (68.8 (24.3-122.7) vs. 17.3 (8.2-56.0), p=0.012), PCT (1.0 (0.3-3.5) vs. 0.1 (0.1-0.4), p<0.001), IL-6 (50.7 (9.5-303.5) vs. 6.5 (3.4-11.4), p=0.010) were higher. In this study, SOFA score, APACHE II score and SLEDAI were counted separately for the survivors and non-survivors, and the results showed that all three scores were significantly different between the two groups (p<0.001). In terms of treatment course, the non-survivor group had more patients undergoing tracheal intubation (21 (67.7%) vs. 2 (7.4%), p<0.001) and CRRT (15 (48.4%) vs. 4 (14.8%), p=0.007), longer ventilator use (77.0 (24.0-191.0) vs. 0.0 (0.0-0.0), p<0.001), and longer ICU stay (8.0 (4.0-14.0) vs. 2.0 (0.0-13.0), p=0.030).

3.3 Immunocytological characteristics

Table 2 demonstrates the immunocytological indices, including the percentage and absolute values of T lymphocytes, B lymphocytes and NK lymphocytes. Compared to the survivors, the non-survivors had lower absolute values of T cells (197.3 (145.2-414.8) vs. 467.9 (206.8-704.7), p=0.032), CD4+ T cells (83.4 (49.0-138.0) vs. 185.5 (86.8-360.3), p=0.005) and CD8+ T cells (111.3 (58.0-242.9) vs. 200.0 (139.8-382.8),

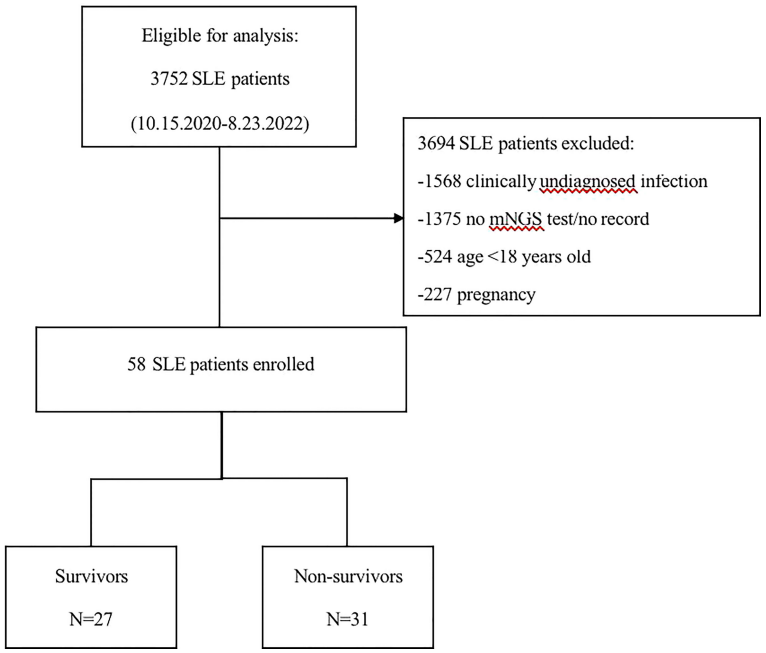

FIGURE 1
Flow chart of analyzed patients. SLE, systemic lupus erythematosus; mNGS, metagenomic next-generation sequencing.

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TABLE 1 Baseline characteristics of SLE patients with co-infection.

Variables	Total (n=58)	Survivors (n=27)	Non-survivors (n=31)	*P* Value
Male, (n%)	12 (20.7)	6 (22.2)	6 (19.4)	0.788
Age, (y)	43.6 ± 15.6	42.0 ± 16.1	44.9 ± 15.3	0.485
BMI, (Kg/M^2)	21.9 (20.1-24.4)	21.9 (20.1-24.5)	21.7 (20.0-23.3)	0.700
History of smoking, (n%)	7 (12.3)	4 (14.8)	3 (10.0)	0.882
History of drinking, (n%)	6 (10.5)	3 (11.1)	3 (10.0)	1.000
Duration of SLE, (y)	2.5 (0.3-7.0)	3.0 (0.6-10.0)	1.0 (0.2-6.0)	0.223
Comorbidities, (n%)				
Hypertension	20 (34.5)	5 (18.5)	15 (48.4)	**0.017**
Type 2 diabetes	6 (10.3)	6 (22.2)	0 (0.0)	**0.006**
Dyslipidemia	36 (64.3)	14 (53.8)	22 (73.3)	0.129
Combined with other AD	9 (15.5)	4 (14.8)	5 (16.1)	1.000
SLE-related symptoms, (n%)				
Renal impairment	34 (58.6)	10 (37.0)	24 (77.4)	**0.002**
Neurological manifestations	25 (43.1)	5 (18.5)	20 (64.5)	**<0.001**
Cardiovascular manifestations	31 (53.4)	11 (40.7)	20 (64.5)	0.070
Multiplasmatic cavity effusion	27 (46.6)	8 (29.6)	19 (61.3)	**0.016**
Gastrointestinal manifestations	39 (67.2)	12 (44.4)	27 (87.1)	**<0.001**
Hematologic manifestations	51 (87.9)	21 (77.8)	30 (96.8)	0.070
Dry syndrome	3 (5.2)	1 (3.7)	2 (6.5)	1.000
Eye manifestations	1 (1.7)	0 (0.0)	1 (3.2)	1.000
Thrombosis	19 (32.8)	6 (22.2)	13 (41.9)	0.111
Types of Clinical Presentation				
≥ 5 kinds	26 (44.8)	4 (14.8)	22 (71.0)	**<0.001**
<5 kinds	32 (55.2)	23 (85.2)	9 (29.0)	
Medication History, (n%)				
Immunosuppressants	41 (71.9)	22 (81.5)	19 (63.3)	0.128
Biological agents	5 (8.6)	3 (11.1)	2 (6.5)	0.872
Laboratory examinations				
White blood cell, ($10^9/L$)	7.2 (5.0-10.5)	6.5 (4.8-9.0)	7.9 (5.9-12.4)	0.107
Hemoglobin, (g/L)	92.0 ± 19.3	98.1 ± 16.3	86.8 ± 20.5	**0.026**
Platelet, ($10^9/L$)	134.1 ± 84.9	181.3 ± 82.1	93.0 ± 64.0	**<0.001**
Glucose, (mmol/L)	6.6 (5.2-9.4)	5.2 (3.8-7.0)	7.8 (6.0-11.0)	**<0.001**
Potassium, (mmol/L)	3.9 ± 0.5	4.0 ± 0.7	3.8 ± 0.4	0.195
Creatinine, (μmmol/L)	78.5 (53.7-155.5)	68.0 (50.0-154.6)	83.0 (58.0-158.0)	0.350
Alanine transaminase, (U/L)	18.0 (10.5-42.5)	15.0 (7.0-41.0)	19.0 (14.0-44.0)	0.145
Aspartate transaminase, (U/L)	32.0 (16.8-53.5)	25.0 (14.0-38.0)	44.0 (18.0-61.0)	0.052
Alkaline phosphatase, (U/L)	72.0 (55.0-116.5)	69.0 (52.0-129.0)	72.0 (56.0-116.0)	0.749
Albumin, (g/L)	28.2 (23.8-32.8)	31.9 (27.9-34.5)	24.7 (22.1-28.6)	**<0.001**
Globulin, (g/L)	27.9 (22.8-31.9)	27.0 (20.4-32.0)	28.2 (24.8-31.5)	0.374

(Continued)

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TABLE 1 Continued

Variables	Total (n=58)	Survivors (n=27)	Non-survivors (n=31)	*P* Value
Total bilirubin, (μmmol/L)	8.2 (4.7-16.1)	5.8 (3.9-8.9)	12.5 (7.3-24.5)	**<0.001**
ΔPH	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	**0.009**
Lactate, (mmol/L)	1.5 (1.0-2.0)	1.1 (0.7-1.4)	1.7 (1.2-2.8)	**0.006**
PT, (s)	11.4 (10.6-12.4)	11.3 (11.0-12.2)	11.9 (10.3-13.1)	0.743
APTT, (s)	29.7 (25.5-35.8)	29.3 (24.9-33.6)	30.3 (25.6-36.5)	0.282
D-dimer, (mg/L)	2.0 (0.6-3.5)	1.0 (0.3-1.9)	3.3 (0.8-4.8)	**<0.001**
LDH, (U/L)	425.0 (278.3-768.0)	372.0 (231.0-460.0)	581.0 (352.0-791.0)	**0.003**
CKMB, (U/L)	9.3 (4.2-19.5)	9.0 (4.4-14.1)	10.9 (3.7-24.4)	0.492
CRP, (mg/L)	39.4 (10.6-93.0)	17.3 (8.2-56.0)	68.8 (24.3-122.7)	**0.012**
ESR, (mm/h)	35.5 (16.0-69.3)	46.0 (24.5-70.5)	27.0 (11.0-74.5)	0.131
PCT, (ng/mL)	0.3 (0.1-2.4)	0.1 (0.1-0.4)	1.0 (0.3-3.5)	**<0.001**
IL-2, (pg/mL)	1.8 (1.0-2.8)	1.6 (1.0-2.2)	1.8 (0.9-3.3)	0.582
IL-6, (pg/mL)	11.4 (5.2-80.2)	6.5 (3.4-11.4)	50.7 (9.5-303.5)	**0.010**
IL-10, (pg/mL)	4.8 (2.6-8.3)	4.8 (2.1-7.9)	5.2 (2.8-9.2)	0.553
IFN-γ, (pg/mL)	1.1 (0.7-4.3)	1.3 (0.8-5.6)	1.1 (0.6-6.0)	0.866
Complement C3, (g/L)	0.7 ± 0.3	0.8 ± 0.4	0.5 ± 0.3	**0.002**
IgG, (g/L)	12.2 (9.2-15.2)	12.3 (7.7-14.6)	12.0 (9.2-15.7)	0.748
Maximum body temperature, (°C)	39.7 (38.8-40.2)	39.5 (37.9-40.2)	39.9 (39.0-40.2)	0.169
Severity score				
SOFA Score	6.0 (1.0-9.3)	1.0 (0.0-3.0)	9.0 (7.0-12.0)	**<0.001**
APACHE II Score	16.0 (8.0-23.0)	8.0 (5.0-13.0)	20.0 (17.0-31.0)	**<0.001**
SLEDAI, (n%)				
Mild activity	13 (22.4)	13 (48.1)	0 (0.0)	**<0.001**
Moderate activity	13 (22.4)	12 (44.4)	1 (3.2)	
Heavy activity	32 (55.2)	2 (7.4)	30 (96.8)	
Treatment measures				
Tracheal intubation, (n%)	23 (39.7)	2 (7.4)	21 (67.7)	**<0.001**
Tracheotomy, (n%)	2 (3.4)	1 (3.7)	1 (3.2)	1.000
Ventilator use time, (h)	8.8 (0.0-104.3)	0.0 (0.0-0.0)	77.0 (24.0-191.0)	**<0.001**
CRRT, (n%)	19 (32.8)	4 (14.8)	15 (48.4)	**0.007**
Prophylactic use of antibiotics, (n%)	50 (86.2)	23 (85.2)	27 (87.1)	1.000
ICU stay, (d)	6.6 (1.9-13.0)	2.0 (0.0-13.0)	8.0 (4.0-14.0)	**0.030**
Hospital stay, (d)	14.0 (8.0-24.3)	15.0 (10.0-27.0)	14.0 (8.0-20.0)	0.265
ICU to hospital stay ratio, (%)	48.7 (14.1-100.0)	20.7 (0.0-49.3)	90.0 (36.1-100.0)	**<0.001**
Days of antibiotic use, (d)	12.0 (7.8-22.0)	13.0 (8.0-27.0)	10.0 (7.0-19.0)	0.322

The data was shown as the mean ± SD, median (interquartile 25-75) or n (percentage). P values in bold meant significantly different (P<0.05). SLE, systemic lupus erythematosus; BMI, body mass index; AD, autoimmune diseases; ΔPH, absolute value of the difference between PH and 7.40; PT, prothrombin time; APTT, activated partial thromboplastin time; LDH, lactate dehydrogenase; CKMB, creatine kinase isoenzyme; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PCT, procalcitonin; IL-2, interleukin-2; IL-6, interleukin-6; IL-10, interleukin-10; IFN-γ, interferon-γ; IgG, immunoglobulin G; SOFA, sequential organ failure assessment; APACHE, acute physiology and chronic health evaluation; SLEDAI, systemic lupus erythematosus disease activity index; CRRT, continuous renal replacement therapy; ICU, intensive care unit.

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TABLE 2 Immunocytological indices in SLE patients with co-infection.

Variables	Total (n=58)	Survivors (n=27)	Non-survivors (n=31)	*P* Value
T cell percentage, (%)	74.1 (67.5-85.0)	81.6 (62.3-87.0)	71.3 (67.7-79.6)	0.251
$CD4^+$ T cell percentage, (%)	29.2 ± 11.4	30.0 ± 10.6	28.4 ± 12.2	0.651
$CD8^+$ T cell percentage, (%)	42.2 ± 17.1	43.3 ± 17.5	41.1 ± 17.0	0.680
B cell percentage, (%)	12.7 (3.7-22.4)	9.2 (1.8-16.8)	18.8 (9.5-23.8)	0.076
NK cell percentage, (%)	7.3 (3.5-11.0)	7.9 (4.5-11.0)	6.4 (2.8-11.6)	0.358
T cell absolute count, (/μL)	275.0 (149.5-616.3)	467.9 (206.8-704.7)	197.3 (145.2-414.8)	**0.032**
$CD4^+$ T cell absolute count, (/μL)	121.9 (56.7-205.3)	185.5 (86.8-360.3)	83.4 (49.0-138.0)	**0.005**
$CD8^+$ T cell absolute count, (/μL)	159.0 (81.0-358.5)	200.0 (139.8-382.8)	111.3 (58.0-242.9)	**0.028**
B cell absolute count, (/μL)	50.3 (18.2-96.2)	37.0 (16.8-125.4)	55.3 (21.7-89.0)	0.808
NK cell absolute count, (/μL)	24.2 (10.4-67.4)	39.0 (20.0-82.2)	21.6 (7.0-33.9)	0.060

The data was shown as the mean ± SD or median (interquartile 25-75). P values in bold meant significantly different (P<0.05). SLE, systemic lupus erythematosus; NK cell, natural killer cell.

p=0.028). However, there was no significant difference in the percentage of each lymphocyte between the two groups (p>0.05).

3.4 Infection type distribution

All 58 patients in this study were diagnosed with clinical infections, and Figure 2 demonstrates the distribution of infection types in all patients. Of the patients, 56 were diagnosed with severe pneumonia or pulmonary infection, 21 with bloodstream infection, 6 with intracranial infection, 4 with gastrointestinal infection, 2 with abdominal infection, and finally, 1 each with leg and foot infection. The pie chart shows that pulmonary infections were the most common, with more than half of the patients having pulmonary infections. Of these, 30 patients had pulmonary infections alone and 17 patients had pulmonary infections combined with bloodstream infections. 26 patients presented with more than one lesion infection.

3.5 Microorganism distribution

Figure 3 shows all microorganisms detected by mNGS and CMT. Overall, mNGS was positive in 47 tests, detecting 62 bacteria, 42 fungi, 66 viruses and 3 mycoplasmas, while CMT was positive in 37 tests, detecting 29 bacteria, 7 fungi and 17 viruses. The bacteria detected by mNGS were *Enterococcus faecium* (n=8), *Acinetobacter baumannii* (n=7), *Pseudomonas aeruginosa* (n=7), *Klebsiella pneumoniae* (n=4), *Mycobacterium tuberculosis complex* (n=4), *Staphylococcus aureus* (n=3), *Haemophilus influenzae* (n=3), *Leuconostoc lactis* (n=2), *Streptococcus pneumoniae* (n=2), *Corynebacterium striatum* (n=2), *Haemophilus parainfluenzae* (n=2), *Enterococcus faecalis* (n=1), *Staphylococcus haemolyticus* (n=1), *Streptococcus salivarius* (n=1), *Lactobacillus crispatus* (n=1), *Lactobacillus salivarius* (n=1), *Tropheryma whipplei* (n=1), *Human Staphylococcus* (n=1), *Nocardia farcinica* (n=1), *Streptococcus milleri* (n=1), *Onion Burkholderia* (n=1), *Stenotrophomonas maltophilia* (n=1), *Legionella pneumophila* (n=1), *Klebsiella aerogenes*

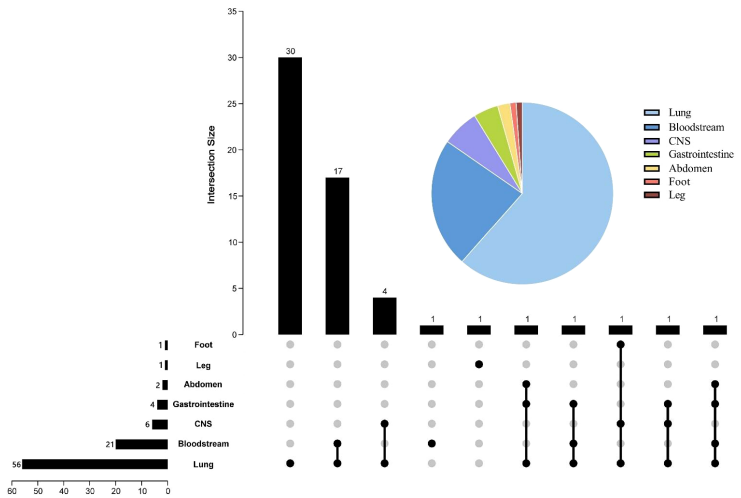

FIGURE 2
Pie and Upset charts of the distribution of infection sites. The pie chart in the upper right corner represents the percentage distribution of each infection site. In the Upset diagram, the rows represent the site of infection, and the number in front of each row indicates the total number of cases of infection at that site; the columns indicate the number of cases for each condition, and the number at the beginning of each column indicates the case of infection at the location of the "black dot"; the black dots connected by lines indicate the presence of multiple infections. CNS, central nervous system.

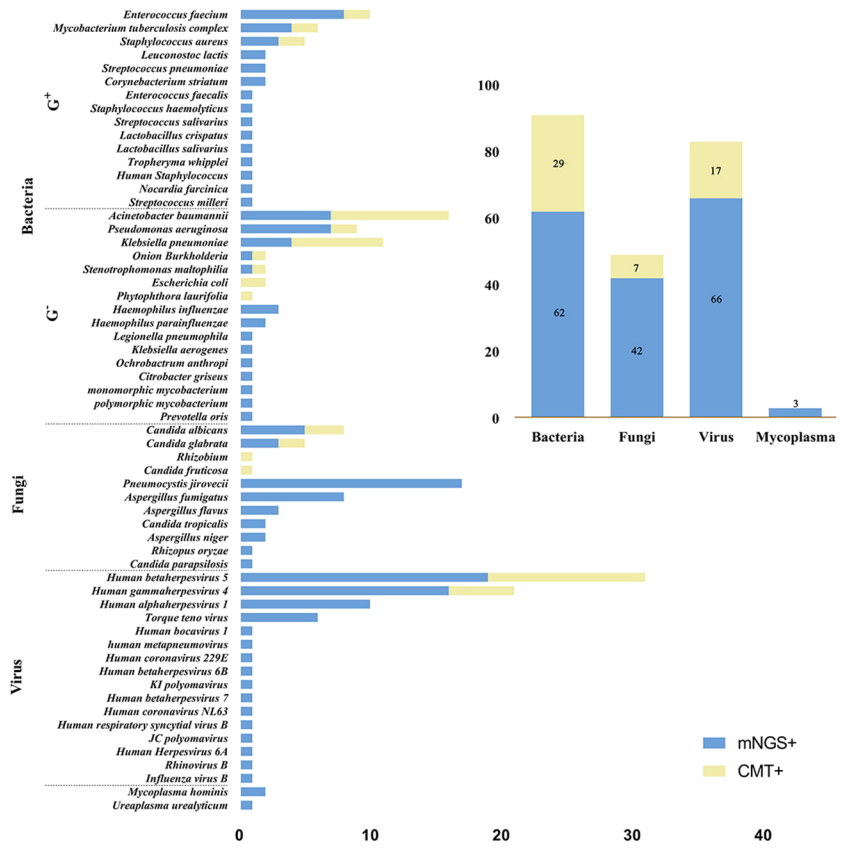

FIGURE 3
Microbial distribution bar charts. The left bar chart shows the distribution of all microorganisms detected by mNGS and CMT, and the top right bar chart shows the distribution of each type of microorganism. mNGS, metagenomic next-generation sequencing; CMT, conventional microbiological tests; G^+, Gram-positive bacteria; G^-, Gram- negative bacteria.

(n=1), *Ochrobactrum anthropic* (n=1), *Citrobacter griseus* (n=1), *Monomorphic mycobacterium* (n=1), *Polymorphic mycobacterium* (n=1), *Prevotella oris* (n=1); the fungi were *Pneumocystis jirovecii* (n=17), *Aspergillus fumigatus* (n=8), *Candida albicans* (n=5), *Candida glabrata* (n=3), *Aspergillus flavus* (n=3),*Candida tropicalis* (n=2), *Aspergillus niger* (n=2), *Rhizopus oryzae* (n=1), *Candida parapsilosis* (n=1); and viruses were *Human betaherpesvirus 5* (n=19), *Human gammaherpesvirus 4* (n=16), *Human alphaherpesvirus 1* (n=10), *Torque teno virus* (n=6), *Human bocavirus 1* (n=1), *Human metapneumovirus* (n=1), *Human coronavirus 229E* (n=1), *Human betaherpesvirus 6B* (n=1), *KI polyomavirus* (n=1), *Human betaherpesvirus 7* (n=1), *Human coronavirus NL63* (n=1), *Human respiratory syncytial virus B* (n=1), *JC polyomavirus* (n=1), *Human Herpesvirus 6A* (n=1), *Rhinovirus B* (n=1), *Influenza virus B* (n=1); mycoplasmas detected included *Mycoplasma hominis* (n=2) and *Ureaplasma urealyticum* (n=1). While the most frequent organisms detected by CMT were *Acinetobacter baumannii* (n=9), *Klebsiella pneumoniae* (n=7), *Pseudomonas aeruginosa* (n=2), *Escherichia coli* (n=2), *Enterococcus faecium* (n=2), *Mycobacterium tuberculosis complex* (n=2), *Staphylococcus aureus* (n=2), *Onion Burkholderia* (n=1), *Stenotrophomonas maltophilia* (n=1), *Phytophthora laurifolia* (n=1), *Candida albicans* (n=3), *Candida glabrata* (n=2), *Rhizobium* (n=1), *Candida fruticose* (n=1), *Human betaherpesvirus 5* (n=12), *Human gammaherpesvirus 4* (n=5).

Figure 4 shows the number of bacteria, fungi and viruses detected by mNGS in each patient. Among all patients, 10 (17.2%) had bacterial infection only, 3 (5.2%) had fungal infection only, and 6 (10.3%) had viral infection only; 7 (12.1%) had bacterial and fungal "co-infection," 5 (8.6%) had bacterial and viral "co-infection", 4 (6.9%) had fungal and viral "co-infection"; and 13 (22.4%) had both bacterial, fungal and viral infections. In addition, two or more bacterial strains were detected in 16 (27.6%), two or more fungal strains in 10 (17.2%), and two or more viruses in 18 (31.0%).

3.6 Comparison of mNGS and CMT diagnostic performance

Figure 5 shows the results of the respective tests of mNGS and CMT. Since 1 patient did not undergo any CMT, only the results of the remaining 57 patients are analyzed here. Of these, 34 (63.0%) patients were positive for both mNGS and CMT, and 7 (13.0%) patients were negative for both. Overall, the positive rate of mNGS testing was higher than that of CMT (82.5% vs 64.9%, p=0.021). The results of the two tests were analyzed separately in the survivor and non-survivor groups, and there was a significant difference between the positive rates of the two tests for patients in the survivor group

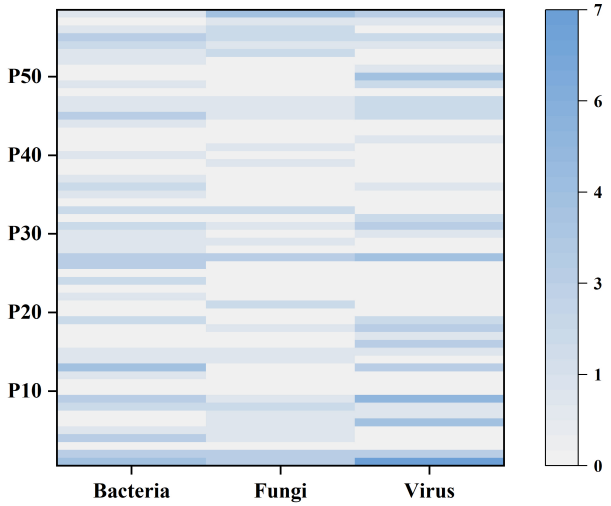

FIGURE 4
The heatmap shows the number of bacteria, fungi and viruses detected by mNGS in each sample. The left y-axis indicates the number of the 58 patients. The shade of color represents the number of microorganisms detected, with larger counts associated with darker colors.

(80.8% vs. 53.8%, p=0.029), while in the non-survivor group there was no significant difference between the two tests (p>0.05). Among the 34 patients who were positive for both mNGS and CMT, we further analyzed the matching of the two test results. Among them, 6 (17.6%) cases were completely matched (exact match between pathogens detected by mNGS and CMT); 19 (55.9%) were partly match (at least one microorganism overlapped between mNGS and CMT); and 9 (26.5%) were mismatch (no pathogen overlap between mNGS and CMT test results).

3.7 Short-term prognosis

The ROC curves of SOFA score, SLEDAI and APACHE II were plotted separately to assess their predictive ability for short-term mortality in co-infected SLE patients, yielding AUCs of 0.956, 0.955 and 0.930, respectively. The specific results are shown in Figure 6 and the legend. The results showed that all three scores accurately predicted short-term mortality in such patients, especially the SOFA score. By calculating the maximum value of the Youden's index, a cut-off point of 6 for the SOFA score and 16 for the APACHE II was determined.

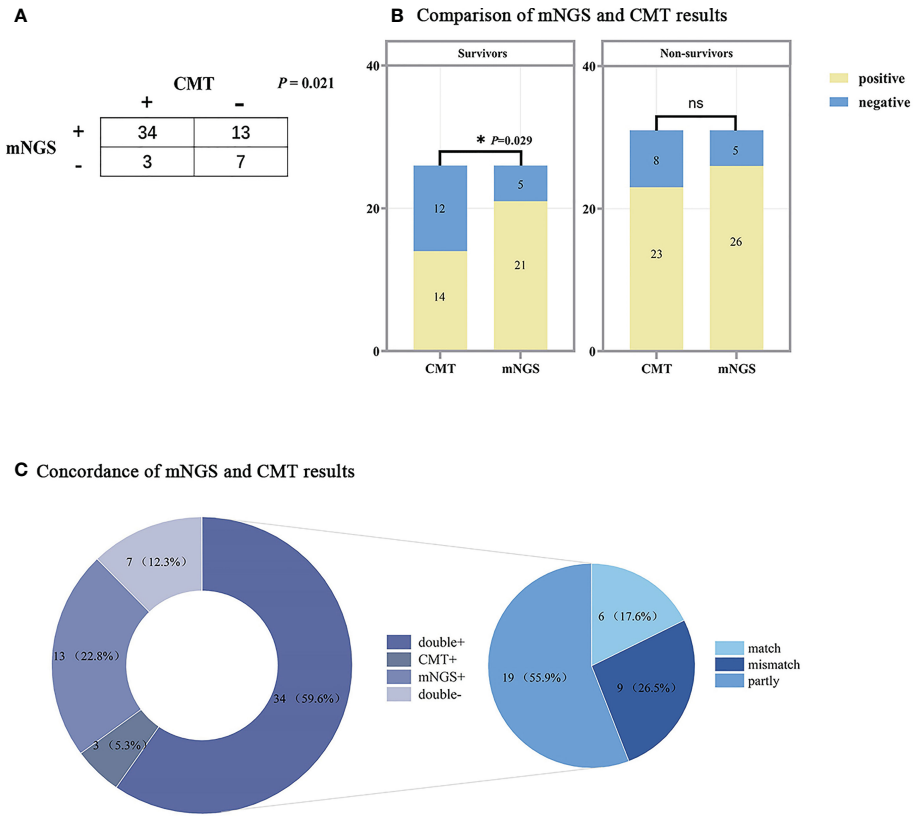

FIGURE 5
Comparison of mNGS and CMT test results. **(A)** Contingency table of the consistency of mNGS and CMT. **(B)** Bar chart of mNGS and CMT assay results in the survivors and non-survivors, the results of the two assays were significantly different in the survivors (*P*<0.05). **(C)** The pie chart shows the distribution of mNGS and CMT results for all patients. The results of the double+ group were further divided into match (6/34), partly match (at least one pathogen confirmed by the other in the test) (19/34), and mismatch (9/34). mNGS, metagenomic next-generation sequencing; CMT, conventional microbiological tests. The following are the details of the three ROC curves: SOFA score: Cut-off value=6; AUC=0.956 (95%CI: 0.905-1.000, P<0.001); Sensitivity: 93.6%; Specificity: 88.9%. SLEDAI: AUC=0.955 (95%CI: 0.893-1.000, P<0.001); Sensitivity: 96.8%; Specificity: 92.6%. APACHE II: Cut-off value=16; AUC=0.930 (95%CI: 0.861-0.998, P<0.001); Sensitivity: 90.3%; Specificity: 88.9%.

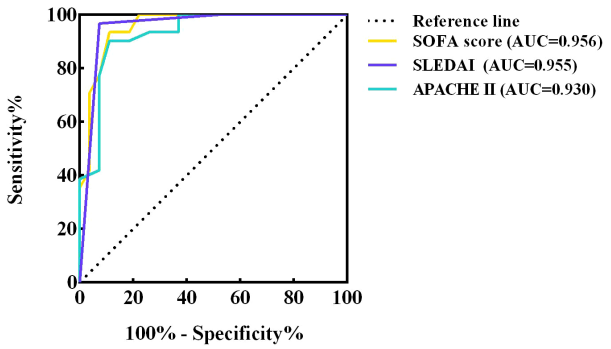

FIGURE 6
ROC curves of SOFA score, SLEDAI and APACHE II. ROC, receiver operating characteristic; AUC, area under curve ROC; CI, confidence interval; SOFA, sequential organ failure assessment; SLEDAI, systemic lupus erythematosus disease activity index; APACHE, acute physiology and chronic health evaluation.

4 Discussion

In recent years, several studies have shown that infection is highly associated with morbidity, hospitalization and mortality in patients with SLE. The European Lupus Project, dedicated to studying the epidemiological features as well as the clinical manifestations of SLE, found that disease activity and infection were the leading causes of death in the first 5 years of illness in patients with lupus (Cervera et al., 2009). The Hopkins Lupus and University College UK cohorts also listed infection as a cause of hospitalization and death (Petri and Genovese, 1992; Goldblatt et al., 2009). disease activity, immune system dysregulation, hormonal and immunosuppression-induced immunodeficiency, and inadmissible damage to organ systems from the disease all contribute to a much higher risk of infection in SLE patients (Rigante et al., 2014).

In this single-center retrospective study, we included 58 patients with coinfected SLE, all of whom were treated with glucocorticoids and 63.3% with cyclophosphamide or mycophenolate. Glucocorticoids have anti-inflammatory and immunosuppressive effects that interfere with leukocyte, fibroblast, and endothelial cell function and reduce the number of circulating monocytes and macrophages, leading to opportunistic infections (Staples et al., 1974; Riccardi et al., 2002). The use of immunosuppressive drugs such as cyclophosphamide may further increase the risk of infection and is still controversial (Gladman et al., 2002; Bosch et al., 2006). In addition to the ability of drugs to cause an increased risk of infection, SLE itself brings irreversible damage to the hematopoietic system, renal function, and immune system that promotes the development of infection. In addition, a significant proportion of SLE patients undergo invasive procedures, such as CRRT placement in patients with lupus nephritis, failure to maintain oxygen saturation requiring tracheal intubation, and deep venous cannulation, all of which have the potential for infection. In the present study, a greater proportion of patients in the non-survivors required tracheal intubation and CRRT, and the ventilator was used for a longer period of time, indicating, on the one hand, that patients in the non-survivors had a higher severity of disease; on the other hand, these operations may have further aggravated the infection and led to worsening of the disease instead. Our analysis of 58 patients with co-infected SLE revealed significant differences in clinical manifestations, laboratory indices, correlation scores and immunocytological indices between the survivor and non-survivor groups. Almost half of the patients in the non-survivors had a history of hypertension, and most of them had renal damage or lupus nephritis. In addition, there were more patients with combined neurological manifestations, multiple plasma membranes and gastrointestinal manifestations, which is consistent with previous studies (Wang et al., 2021). In-hospital death occurred in 71% of patients with ≥5 systems involved in SLE, suggesting that the more diverse the clinical presentation, the higher the mortality may be. In terms of test results, there were also significant differences in several indicators between the two groups, specifically concerning inflammatory indicators such as hemoglobin, platelets, albumin, total bilirubin, complement C3 and CRP and PCT. During clinical treatment, more attention should be paid to changes in these tests, which may affect the outcome of patients with SLE. manderson AP et al. concluded that the complement system hair plays an extremely important role in the pathological process of SLE (Manderson et al., 2004). Complement C3 is central in the classical and bypass pathways of the complement system, and its degradation products C3d and C4d are thought to be markers of complement activation in the inflammatory response (Ricklin et al., 2016; Deng et al., 2022). The circulatory system and endogenous immune complexes cause inflammatory cell infiltration, inflammatory mediator release, cytokine overproduction, and ultimately organ damage with increased complement C3 consumption and decreased production, increasing the risk of infection and exacerbating renal injury (Ho et al., 2001; Qi et al., 2018; Sawada et al., 2019). The predictive value of CRP and PCT for infection in patients with SLE is not yet clearly established. Some studies have suggested that SLE patients can be distinguished from SLE disease activity by determining whether they are infected based on elevated CRP, but some studies have reported conflicting results (Roy and Tan, 2001; Navarro-Zarza et al., 2010). In addition, PCT has been proposed to have a negative predictive value for bacterial infection in active SLE (Singh and Singh, 2020). Therefore, the diagnostic value of inflammatory indicators for infection in SLE patients needs to be confirmed by more studies, but in our study, inflammatory indicators such as CRP, PCT, and IL-6 were significantly elevated in the non-surviving group of patients.

The homeostasis of lymphocyte subsets is important for the immune response (Prado et al., 2013; Katsuyama et al., 2018). T cells are an important factor in the pathogenesis of SLE, and alterations in T cell signaling, cytokine production, and defects in proliferation and regulatory functions have been demonstrated in SLE patients (Crispín et al., 2010; Kaul et al., 2016). T lymphocytopenia, especially CD4+ T lymphocytopenia, is the most common hematological abnormality in SLE (Durand et al., 2000). CD4+ T lymphocytes are closely related to the body's immunity and a decrease in CD4+ T lymphocytes often indicates an increased likelihood of opportunistic infections such as Pneumocystis carinii pneumonia (PCP) or viral infections. In this study, lymphocytes from SLE patients were counted and analyzed, and the absolute values of T

lymphocytes were found to be lower in patients in the non-survivors than in those in the survivors. It is evident that SLE patients commonly experience a decrease in T lymphocytes, but to a greater extent in those with severe disease. However, there was no significant difference in the percentage of T lymphocytes between the two groups, probably because the sample size was too small for the difference to be significant. In the clinical management of SLE, we should pay attention to the above-mentioned tests with significant differences, which are inseparable from the severity of the disease, and emphasize the importance of lymphocyte typing, so that every SLE patient can have this test perfected as much as possible.

In addition to the differential tests, the SOFA, APACHE II, and SLEDAI scores were introduced in this study to investigate whether they could effectively predict the short-term prognosis of SLE patients. The AUC of the SOFA ROC curve was the largest, indicating that it is a good predictor of in-hospital survival in patients with SLE infection.

The mNGS test is used as a new technology for pathogen detection to identify new or unexpected pathogens (Hu et al., 2021). It is capable of sequencing nucleic acids from all organisms in a sample and theoretically has the ability to detect any infectious microorganism (Haslam, 2021). The traditional pathogenic tests that are often used in clinical practice today all have their own limitations. Bacterial/fungal culture assays both have long testing period and low positive rates (Guarner and Brandt, 2011). Viral assays are based on PCR with serological testing, have fixed targets, and are biased (Maartens et al., 2020). Although these traditional assays are the gold standard for pathogen detection and have sufficient evidence-based medical evidence, they are difficult to detect emerging/rare pathogens and have limited sensitivity with narrow detection targets. In contrast, mNGS is now increasingly used in clinical settings because it does not require culture and has a short reporting time, has the ability to detect 27,000 species of bacteria, fungi, viruses and parasites, and can achieve a 30% positive rate. However, due to the high positive rate and the lack of standardized testing criteria, the true and false positivity of microorganisms and the guiding significance for clinical diagnosis have not been fully affirmed. In this study, we analyzed the distribution of infection types in SLE patients and compared the diagnostic performance of mNGS with that of CMT. In terms of infection types, the most common type of infection in SLE patients was pulmonary infection, followed by bloodstream infection and intracranial infection. This differs from the study by Gladman et al, who found that the top three infection types in SLE patients were respiratory, cutaneous, and genitourinary tracts (Gladman et al., 2002). In terms of the types of microorganisms detected, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterococcus faecium* were the most common bacteria, *Pneumocystis jirovecii*, *Candida albicans*, *Aspergillus fumigatus* were the most common fungi, and *Human betaherpesvirus 5* and *Human gammaherpesvirus 4* were the most common viruses. The microorganisms detected by both mNGS and CMT were dominated by bacteria and and viruses. Comparing the diagnostic results of mNGS with those of CMT, we found that the concordance rate was not high, with only 17.6% of the results overlapping completely, especially for the surviving group of patients, where there was a significant difference in the positive rate between the two tests (p=0.029). This indicates that for patients with relatively mild disease and high in-hospital survival rate of SLE infection, the bias in the test results between mNGS and CMT is relatively high; while for patients with severe disease and high mortality rate, the positive rates of the two assays are similar with no significant difference. However, it may also be that the difference is covered by severe multi-organ failure. Therefore, for patients with SLE, we can first score them, and when the SOFA score is ≥6, which means that they have a high in-hospital mortality rate, when we suspect co-infection for pathogenic testing, the efficacy of mNGS and CMT is similar, and one of them is preferred; while when their SOFA score is <6 and they are judged to have a good prognosis, mNGS with a high positive rate is recommended for pathogenic testing The mNGS test with a high positive rate is recommended for patients with a good prognosis with a SOFA score <6. Of course, for patients who are not in an emergency situation and are in a better financial position, it is recommended to improve the mNGS test and CMT to corroborate each other and better guide the treatment.

Several limitations of this study remain. Firstly, this study is a single-center retrospective study with relatively small sample sizes in both the survivor and non-survivor groups, and the results may be biased compared to studies with large sample sizes; secondly, the rigour of the conclusions could be improved as matched patients without mNGS results were not included for comparison; furthermore, due to the lack of criteria for interpreting mNGS results, the mNGS results derived in this study may lead to false positives or false negatives; finally, the majority of patients in this study had been prophylactically administered antibiotics prior to mNGS or CMT, which may have led to changes in the bacterial/fungal/viral profile and affected the pathogenic test results. More prospective and multicenter studies with large samples are needed in the future to explore the infection characteristics of SLE patients and will further investigate the interpretation criteria of mNGS results.

5 Conclusion

In SLE patients with co-infection, great clinical attention should be paid to their clinical manifestations, the diversity of which is closely related to the short-term prognosis of the patients. In such patients, it is important to refine lymphocyte typing, and in this study, SLE patients who experienced in-hospital death had significantly lower absolute T lymphocyte values. the SOFA score, APACHE II, and SLEDAI were all good predictors of the short-term prognosis of co-infected SLE patients. The most relevant prognosis is the SOFA score, where SOFA ≥ 6 means that the patient is much more likely to die in-hospital. mNGS and CMT positivity rates differed significantly in the survivor group, so we recommend that SLE patients with SOFA scores < 6 be tested for mNGS as early as possible. Of course, when economic conditions and practical situations allow, we recommend that the importance of mNGS testing be increased and anti-infective medication be given in conjunction with the actual situation to avoid aggravation or persistence of infection in SLE patients, thus reducing mortality and improving short-term prognosis.

Data availability statement

The data that support the findings of the study "Short-term prognostic analysis of patients with systemic lupus erythematosus co-infection and comparison of mNGS and conventional microbiological test results" have been deposited into EMBL database with accession number PRJEB60557.

Ethics statement

The studies involving human participants were reviewed and approved by The Ethics Committee ofthe First Affiliated Hospital of Zhengzhou University (approval number: 2022-KY-0045). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

XZ, M-XD and L-PB: software, formal analysis, data curation, writing-original draft. X-YZ and XZ: conceptualization, methodology, writing - review and editing. Y-YL: validation, formal analysis. X- YZ and XZ: term, resources, project administration, funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by Science and Technology Development Project of Henan Province (212102310210) for X-YZ and Joint Project of Medical Science and Technology of Henan (LHGJ20190095) for XZ.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Brief Research Report
PUBLISHED 05 April 2023
DOI 10.3389/fcimb.2023.1064760

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 08 October 2022
ACCEPTED 17 March 2023
PUBLISHED 05 April 2023

CITATION
Xiang C, Wu X, Wei Y, Li T, Tang X, Wang Y, Zhang X, Huang X and Wang Y (2023) First report on severe septic shock associated with human Parvovirus B19 infection after cardiac surgery. *Front. Cell. Infect. Microbiol.* 13:1064760. doi: 10.3389/fcimb.2023.1064760

First report on severe septic shock associated with human Parvovirus B19 infection after cardiac surgery

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Background: Human Parvovirus B19 (PB19) is a single-stranded DNA virus. Septic shock from viremia is rare with PB19; however, this infection can progress to life-threatening conditions. We report the first case of severe septic shock associated with a PB19 infection after cardiac surgery.

Case Presentation: A 50-year-old Chinese woman received elective double metal valve replacement, including the aortic valve and the mitral valve, under cardiopulmonary bypass (CPB) and suffered severe septic shock on postoperative day (PD) 30. Through the detection of PB19-specific nucleic acids in blister fluid and serum samples *via* metagenomic next-generation sequencing (mNGS), positive serum PB19 IgM and no other proven infection, acute PB19 infection was confirmed. After five days of combined treatment, no further fever or abdominal discomfort was noted, and the patient's circulation gradually became stable without vasoactive medications.

Conclusion: PB19 may be an unrecognized cause of septic shock, rash, fever of unknown origin or multiple systemic signs and symptoms, especially in immunosuppressed and immunocompetent critically ill patients. Investigations for viral aetiology are needed.

KEYWORDS

human Parvovirus B19, infection, septic shock, cardiac surgery, metagenomic next generation sequencing

1 Introduction

PB19 is a single-stranded DNA virus that causes many clinical disorders, of which the most common are erythema infectiosum (EI), aplastic crisis complicating chronic haemolytic anaemia, self-limiting arthritis, and hydrops foetalis, which lack specificity (Meyer, 2003; Landry, 2016). Septic shock from viremia is rare with PB19; however, this infection can progress to life-threatening conditions. In our extensive review of the literature, only six reports of septic shock were associated with PB19. (Ferraz et al., 2005; Bailey, 2006; Kaur et al., 2011; Panicker et al., 2011; García-Salido et al., 2014; Kuriyama et al., 2016)

The pathogenesis of severe septic shock associated with PB19 is not well understood. PB19 targets erythroid progenitors in the bone marrow by binding to glycosphingolipid globoside, leading to large receptor-induced structural changes triggering cell death either by lysis or by apoptosis mediated by the non-structural 1 protein (NS 1) (Rogo et al., 2014). Transactivation of the IL-6 gene by NS1 may represent a common pathway to parvovirus B19-induced tissue damage in many different sites of the body (Mitchell, 2002). The viral particles or the cellular debris from red blood cell destruction could have initiated the systemic inflammatory activation, resulting in sepsis in this case.

We report the first case of severe septic shock associated with PB19 infection after cardiac surgery, which was successfully treated by immunoglobulin. A review of literature on this situation is also performed.

2 Case presentation

Our patient is a 50-year-old Chinese woman. She was hospitalized for exertional dyspnoea and easily induced fatigue that she experienced over the 17 years prior to the admission. The patient had a prior diagnosis of rheumatic valvular disease. At admission, the chest X-ray showed cardiac enlargement. Cardiac sonography revealed severe aortic valve disease (stenosis and regurgitation) and mitral stenosis. Therefore, double metal valve replacement, including the aortic valve and the mitral valve, was suggested and performed. After the surgical intervention, a transesophageal echocardiogram revealed no residual valvular event; however, hypotension and a rapid pulse rate were still observed after the operation (lowest blood pressure 88/44 mmHg; heart rate 112 beats per minute). Through the use of vasoactive medications, fluid resuscitation, anti-infectives and other treatments, the patient was transferred to the department of cardiac surgery in good condition on PD 7.

Unfortunately, the patient's temperature increased to 38.5°C in the evening of PD 30, and she had shaking chills, diarrhoea, a heart rate of 138 per minute, a respiratory rate of 32 per minute, and a blood pressure of 130/62 mmHg with norepinephrine (0.02 μg/kg/min) infusion. An erythematous rash was present over the trunk and extremities. Persistent fever and diarrhoea developed with persistent circulatory failure, despite empiric antibiotic treatment (piperacillin-tazobactam 4.5 g iv q8 h), which was given after blood culture, stool culture and sputum culture sampling.

Due to further deterioration of the patient's circulation, accompanied by continuous fever (up to 41°C), progressive decrease of haemoglobin, anuria and a worsening of the rash on her limbs, the patient was transferred to the SICU for continuous treatment on PD 32. On the physical examination, the blood pressure was 96/70 mmHg with norepinephrine (0.08 μg/kg/min), temperature 37.3°C, respiratory rate of 30 per minute, oxygen saturation 98% with nasal cannula of oxygen-therapy at 4 litres-min, the heart rate was 140 bpm, and a metal sound of the valve was noted at the left-sternal border without murmur, pulmonary crackles, or oedema in the lower extremities. An erythematous maculopapular rash was distributed on the arms and lower legs but did not involve the trunk (Figure 1A). There were erythematous macules and papules, approximately 1 to 5 mm in diameter, which did not blanch on pressure. Laboratory data included a white blood cell count of 13.53 × 109/L with 93.3% neutrophils and 4.9% lymphocytes, a platelet count of 174 × 109/L, haemoglobin of 82 g/L, blood lactate of 2.1 mmol/L, hypersensitive C-reactive protein (Hs-CRP) of 228.09 mg/L, procalcitonin (PCT) of 332.00 ng/ml, B-type natriuretic peptide of 71.50 pg/ml, blood glucose of 12.11 mmol/L, and arterial blood lactate of 4.2 mmol/L. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), and blood urea nitrogen (BUN) were variably elevated (Table 1). Although the patient had a normal number of B lymphocytes and natural killer cells, the immunologic condition showed decreased counts of CD4+ and CD8+ lymphocytes (CD4+: 135/μl, CD8+: 120/μl).

Bedside transthoracic echocardiography showed that the patient's right ventricle was in normal size and functioned well, and the patient had enlargement of the left atrium and left ventricle (LA: 50 mm; LAD: 53 mm). The LVEF was 55%, without pericardial effusion. No mechanical prosthetic valve dysfunction was observed. The electrocardiograph showed sinus tachycardia without abnormal ST-T.

The detection of mNGS in the blister fluid and serum from this patient was performed. Empiric antibiotic administration (vancomycin, meropenem and caspofungin) was administered. When the patient's haemodynamic status gradually worsened, norepinephrine (0.72 μg/kg/min), terlipressin (0.02 μg/kg/min) and cortisol were given due to the emergence of the situation resulting from haemodynamic instability. To decrease the internal milieu disorder in time and clear the various inflammatory mediums efficiently, continuous renal replacement therapy plus hemoperfusion (HA380) was performed. To identify the pathogen as soon as possible, blood culture, stool culture and sputum culture were obtained before antimicrobial administration was continued.

Interestingly, vesicles appeared on PD 33 and were pale or yellowish from serous fluid matter. The vesicles slowly became enlarged and ruptured, leaving exuding areas (Figure 1B).

After transfer to our department, PB19-specific nucleic acids were detected in mNGS samples of the patient's blister fluid and serum, and the fluids were negative for other viral, fungal, or bacterial nucleic acids (Figure 2). Confirmatory examination showed that PB19 IgM and IgG in serum both were positive (Table 2). We checked the antefebrile serum antibody, when PB19 IgM and IgG were both positive after fever. Interestingly, confirmatory examination of antefebrile serum antibody showed

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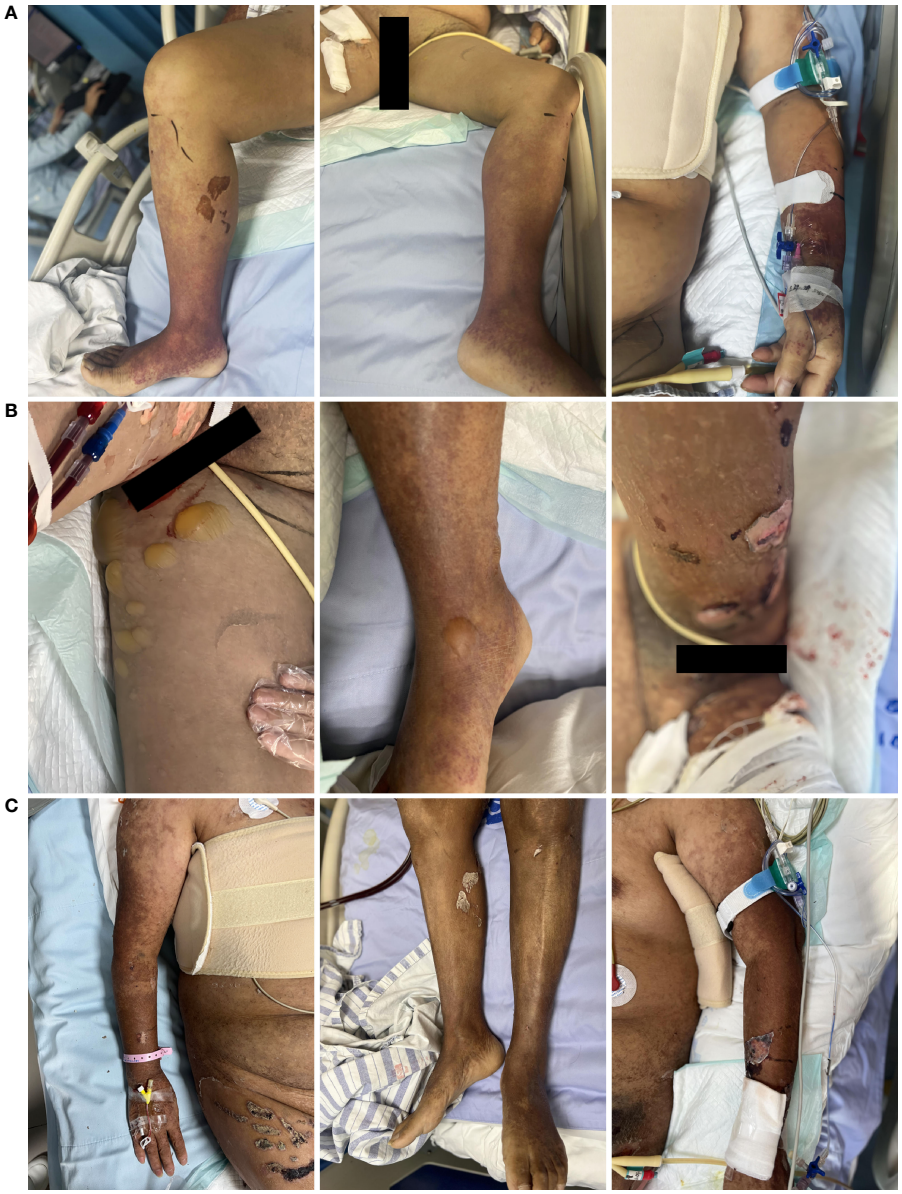

FIGURE 1
Evolution of the rash. **(A)** The early lesions were erythematous macules or papules approximately 1 to 5 mm in diameter that did not blanch on pressure. The erythematous maculopapular rash was mostly distributed on the arms and lower legs but was less distributed in the trunk. **(B)** The vesicles were pale or yellowish from serous fluid matter. The vesicles slowly become enlarged and ruptured, leaving exuding areas. **(C)** Skin healing with desquamation.

that PB19 IgG in serum was positive, while IgM was negative (Table 2). All of the cultures, including blood, stool and sputum, obtained negative results. The serology of HIV, HBV, HCV, EBV, CMV, *Toxoplasma gondii* and *Mycoplasma pneumoniae* were all negative. Laboratory investigations revealed a negative serum (1,3)-β-D-glucan test and galactomannan antigen test. The hypocomplementemia complement component 3 concentration was 0.44 [normal 0.9–1.8] g/L; the complement component 4 concentration was 0.08 [normal 0.100–0.400] g/L). Serologic testing showed elevated immunoglobulin E (408.00 [normal 0.00–100.00] IU/mL), but the levels of the other immunoglobulins were normal. The autoimmune antibody profile and pemphigus antibodies were within normal limits. Based on these clinical findings, a multidisciplinary consultation was organized. We substituted vancomycin, meropenem and caspofungin with 4.5 g of intravenous piperacillin-tazobactam every 8 hours. On this basis, this patient was treated with intravenous immunoglobulin (IVIG) 400 mg/kg/d for 5 consecutive days and methylprednisolone 1 mg/kg/d. Topical measures were performed to prevent secondary infections and facilitate the healing of the blisters. After five days of the combined treatment, no further fever or abdominal discomfort was noted, and the patient's circulation gradually

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TABLE 1 The variations trend of laboratory data.

	unit	On admission	PD* 29	PD 30♯	PD 31	PD 32	PD 33	PD 34	PD 35	PD 36	PD 37	PD 38	PD 39	reference range
White blood cell	10^9/L	7.070			9.630	11.610	6.350	11.370	11.620	7.450	8.410	7.660	7.200	3.50-9.50
Neutrophil	10^9/L	5.182			7.271	11.146	5.632	9.994	11.027	6.541	7.207	6.787	5.530	1.80-6.30
Neutrophil ratio	%	73.3			75.5	96.0	88.7	87.9	94.9	87.8	85.7	88.6	76.8	40.0-75.0
Lymphocyte	10^9/L	1.188			1.348	0.302	0.508	0.955	0.256	0.410	0.690	0.421	0.482	1.10-3.20
Hemoglobin	g/L	114			80	79	64	62	70	66	74	77	85	115-150
Platelets	10^9/L	174			343	193	71	56	36	30	30	30	54	101-320
PCT	ng/mL	0.03			0.27	330.40	160.00			43.30			8.74	0-0.05
Hs-CRP	mg/L	6.41			32.36	151.76	208.39	193.01	100.35	120.93	77.89	51.11	29.34	0-5.00
Total bilirubin	umol/l	17.7	11.2		10	11.2	9.9	18.3	25.7	20.2	15.3	13.4	17.1	0.0-21.0
Direct bilirubin	umol/l	5.3	3.6		1.9	8.4	5.8	9.7	15.1	11.5	8.4	8.9	6.6	0.0-8.0
AST	U/L	61	26		30	171	132	133	185	138	64	29	29	13-35
ALT	U/L	106	22		20	57	59	61	79	69	52	37	30	7-40
Cr	umol/l	58.6	78.0		87.0	338.0	320.0	271.0	208.0	211.0	316.0	364.0	374.0	49.0-82.0
BUN	mmol/l	5.55	8.10		7.80	15.10	13.80	13.00	12.10	13.30	31.70	37.70	38.00	2.60-7.50

*PD, postoperative day; ♯ The patient suffered the symptoms of unknown infection at in the evening of PD 30, like fever, shaking chill, diarrhea subsequently.

stabilized without vasoactive medications (Figure 3) (Gaies et al., 2010). The skin healed with desquamation (Figure 1C).

3 Discussion

PB19 infection is usually a self-limited disease (Landry, 2016). Due to the difficulty in isolating the virus by conventional cell culture, the diagnosis of PB19 relies on serology and DNA detection (Young and Brown, 2004; Landry, 2016). The evidence supporting acute PB19 infection in our case was derived from the presence of PB19-specific nucleic acids (in serum and blister fluid), positive serum PB19 IgM and no other proven infection (Miron et al., 2006; Landry, 2016). Although PB19 acute infections of children can result in EI, up to 50% infection is asymptomatic. A prodromal nonspecific illness consisting of fever, chills, headache, malaise, and myalgias, coinciding with PB19 viremia,

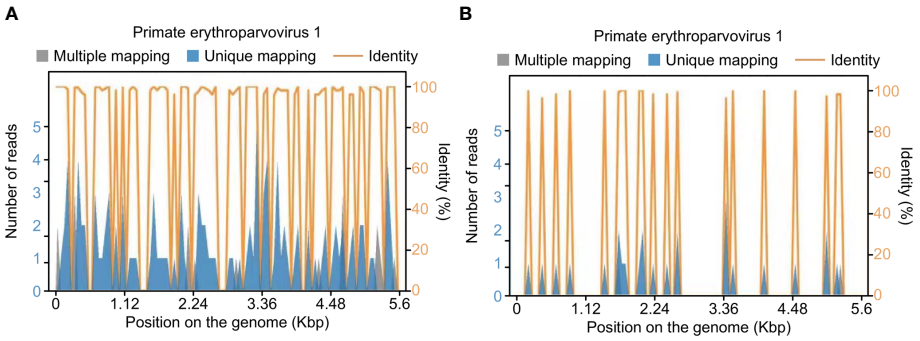

FIGURE 2
Human Parvovirus B19-specific nucleic acids were detected in mNGS samples of serum and blister fluid. Sequence distribution map: This part only shows the sequence distribution map of species with ≥3 nonrepeat specific sequences detected. **(A)** Serum samples. The total length covered on the genome is 3623 bp, the coverage is 64.7427%, and the average depth is 1.65X. **(B)** Blister fluid samples. The total length covered on the genome is 1059 bp, the coverage is 18.9242%, and the average depth is 1.18X.

TABLE 2 The changes of IgM and IgG in serum.

	IgM	IgG	Normal range	test method
PD 28#	-	+	-	ELISA
PD 33*	+	+	-	ELISA

28 days after operation; *33 days after operation and 4 days after fever.

can occur, followed by a typical "slapped cheek" facial rash and a lacy, reticular erythematous rash on the trunk and extremities coincident with the immune response. (Landry, 2016) But in adult patients, the typical EI rash is much less common and primary PB19 infection is associated with polymorphous skin manifestations with four predominant, sometimes overlapping, patterns:exanthema, which was reticulated and annular in some cases; the gloves-and-socks pattern; the periflexural pattern; and palpable purpura. Concomitantly, the rash predominated on the legs, trunk, and arms, with a lower frequency of facial involvement. (Drago et al., 2014; Mage et al., 2014). Likewise, at an earlier stage of the disease, the gloves-and-socks pattern and palpable purpura were observed on the lower legs, trunk, and arms without pruritus in our case. And these lesions gradually became erythematous with vesiculation.

Septic shock from viremia rarely occurs with PB19; however, this infection can progress to life-threatening conditions, as seen in our patient. This is the first report on severe septic shock associated with PB19 infection after cardiac surgery. In our extensive review of the literature, only six reports of septic shock were associated with PB19 (Ferraz et al., 2005; Bailey, 2006; Kaur et al., 2011; Panicker et al., 2011; García-Salido et al., 2014; Kuriyama et al., 2016). It is noteworthy that there was only one published case in which the patient had sickle cell disease, which is associated with an increased risk of serious and life-threatening infections (Panicker et al., 2011). Most of the patients reported in the case reports were previously healthy, including 3 adult women and 7-year-olds. In contrast, the reported patient's immunity level was low at the time of hospitalization after cardiac surgery, which might be due to cardiac surgery injury, CPB, blood loss and malnutrition (Angele and Faist, 2002; Gaudriot et al., 2015; Candela et al., 2021). A previous study showed that the bactericidal activity of neutrophil bactericidal agents against *Staphylococcus aureus* was decreased after cardiac surgery (Mekontso-Dessap et al., 2005). Chalk et al. (2013) have also reported dysfunction of pulmonary macrophages, and patients have strong systemic immune depression after CPB. Moreover, Torrance et al. (2018) reported an association between monocyte dysfunction and major surgery in a study, consisting of 119 participants undergoing the elective gastro-intestinal surgery. As a result, immunodepression increased the susceptibility to infection in surgical patients (Angele and Faist, 2002; Volk, 2002). Findings from the Cardiac Surgical Intensive Care Unit records, which were conducted among 6864 patients after cardiac surgery in 2013-2014, indicate that 4.6% of the patients developed nosocomial infections, including 24 (7.5%) that are consistent with the diagnosis of bloodstream infection (Sahu et al., 2016). Klebsiella, Pseudomonas, Enterobacter, *Escherichia coli*, Acinetobacter, and Staphylococcus were the most frequent pathogens isolated from patients with bloodstream infections (Sahu et al., 2016).

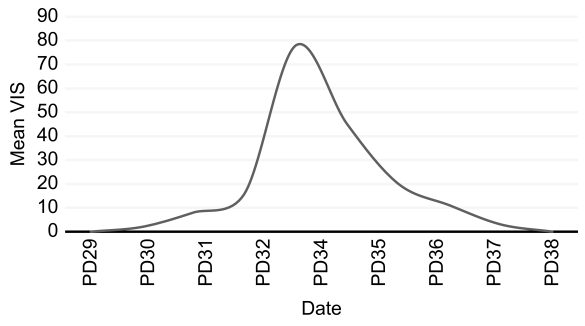

FIGURE 3
The trend of the mean VIS. #PD, postoperative day; *VIS, Vasoactive–Inotrope Scoring. VIS= dopamine dose (μg/kg/min) + dobutamine dose (μg/kg/min) + 100* epinephrine dose (μg/kg/min) + 10*milrinone dose (μg/kg/min) + 10,000* vasopressin dose (U/kg/min) + 100* norepinephrine dose (μg/kg/min). The hourly doses of the following inotropic and vasoactive medications were recorded every day: dopamine, dobutamine, epinephrine, norepinephrine, milrinone, and vasopressin. The mean VIS was obtained by averaging the hourly scores during 24-hr periods.

However, the pathogenesis of severe septic shock associated with PB19 is not well understood. PB19 targets erythroid progenitors in the bone marrow by binding to glycosphingolipid globoside, leading to large receptor-induced structural changes triggering cell death either by lysis or by apoptosis mediated by the non-structural 1 protein (NS 1) (Rogo et al., 2014). Transactivation of the IL-6 gene by NS1 may represent a common pathway to parvovirus B19-induced tissue damage in many different sites of the body (Mitchell, 2002).

Despite the patient's immunocompromised status, it remains unclear how the reported patient became infected. As previously reported in the literature, in compromised hosts, B19 infection can be acquired *via* the respiratory route, from endogenous reactivation, or from blood products (Landry, 2016). No sign revealing cross infections was found in our case, although nosocomial outbreaks of PB19 infection have been reported (Pillay et al., 1992; Seng et al., 1994; Miyamoto et al., 2000; Lui et al., 2001). Strong evidence reveals that PB19 DNA has been detected in bone marrow samples, peripheral blood, synovium, myocardium, and skin for months and even years after the primary infection and the resolution of symptoms of normal hosts (Cassinotti et al., 1993; Kerr et al., 1995; Cassinotti et al., 1997; Cassinotti and Siegl, 2000; Heegaard et al., 2002; Hokynar et al., 2002; Söderlund-Venermo et al., 2002; Schenk et al., 2009). In addition to *de novo* infections, viruses go into latency periods and can be reactivated in both immunosuppressed and immunocompetent critically ill patients (Landry, 2016; Fragkou et al., 2021). The infection in this patient may be explained by secondary reactivation

of PB19 due to the patient's immunocompromised status. The evidence supporting this hypothesis in our case was derived from the positive serum PB19 IgG, while IgM was negative on PD28 (2 days before fever). IgG is a protective antibody, which indicates the past infection. Additionally, the transmission of symptomatic PB19 infection by blood products used during surgery, such as fibrin sealant, has been reported previously (Hino et al., 2000; Enoki et al., 2002; Kawamura et al., 2002; Tournoux et al., 2004). The International Hemovigilance Network collected aggregate data on suspected transfusion-transmitted infections for 2013-2016 from member national hemovigilance systems. In one patient, there was clinical suspicion of PB19 infection obtained by blood transfusion (Politis et al., 2019).

Unfortunately, no antiviral therapy is available to treat PB19 infection (Rogo et al., 2014; Landry, 2016; Fragkou et al., 2021). The treatment of choice mainly includes red-cell transfusion, adjustment in medications to restore or improve the patient's immune system, and administration of IVIG (Szenborn, 2011; Landry, 2016). A regimen of 400 mg/kg/day for five to ten days has been suggested (Young and Brown, 2004). However, the efficiency of IVIG in severe septic shock patients is unclear. Because the patient's circulation gradually stabilized with clinical improvements after treatments, we believe that IVIG was beneficial in this case.

In our case, there is a limitation, which should be mentioned. Serum procalcitonin was markedly elevated, and there might be other unrecognized infections, although mNGS, all of the cultures, including blood, stool and sputum, and serology of HIV, HBV, HCV, EBV, CMV, Toxoplasma gondii and Mycoplasma pneumoniae obtained negative results except PB19.

The case presented provides evidence that PB19 may be an unrecognized cause of septic shock, rash, fever of unknown origin or multiple systemic signs and symptoms, especially in immunosuppressed and immunocompetent critically ill patients. Investigations for viral aetiology are needed.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

Author contributions

YPW designed the study. CLX, XXW, and YPW contributed to collection and collation the clinical data. CLX and YKW contributed to extract data from literature and manuscript writing. TLL, XMT, XQZ, XBH and YW contributed to manuscript revisions and approved the final manuscript. All authors contributed to the article and approved the submitted version.

Funding

Funding was provided by the Key Research and Development Project of Science and Technology of Sichuan Province (Grant no. 21ZDYF4270), Sichuan Science and Technology Department (Grant no. 2021YFS0373).

Acknowledgments

The authors wish to thank the patient for participating in this study.

Conflict of interest

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

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 17 April 2023
DOI 10.3389/fcimb.2023.1146088

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Clinical Microbiology, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 16 January 2023
ACCEPTED 28 March 2023
PUBLISHED 17 April 2023

CITATION
Zhao X, Bai L-P, Li B-Y, Yue Z-Z, Zhao Y-C and Zhao X-Y (2023) Comparison of mNGS and conventional culture in non-organ transplant critically ill patients supported by ECMO: a single-center study.
Front. Cell. Infect. Microbiol. 13:1146088.
doi: 10.3389/fcimb.2023.1146088

Comparison of mNGS and conventional culture in non-organ transplant critically ill patients supported by ECMO: a single-center study

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Objectives: Infection is one of the important causes of death in intensive care unit (ICU) patients. At present, there are few articles focused on the detailed analysis of pathogenic microorganisms detected in different therapy periods of critically ill patients supported by extracorporeal membrane oxygenation (ECMO).

Methods: From October 2020 to October 2022, ECMO-assisted patients who underwent multiple times of both metagenomic next-generation sequencing (mNGS) test and conventional culture were enrolled continuously in the First Affiliated Hospital of Zhengzhou University. The baseline data, laboratory test results, and pathogenic microorganisms detected by mNGS and traditional culture in different time periods were recorded and analyzed.

Results: In the present study, 62 patients were included finally. According to whether the patients survived at discharge, they were divided into the survivor group (n = 24) and the non-survivor group (n = 38). Then, according to the different types of ECMO support, they were divided into the veno-venous ECMO (VV ECMO) group (n = 43) and the veno-arterial ECMO (VA ECMO) group (n = 19). The summit period of specimens of traditional culture and mNGS detection of ECMO patients was 7 days after admission, and the largest number of specimens of surviving patients appeared after ECMO withdrawal. The total number of traditional culture specimens was 1,249, the positive rate was 30.4% (380/1,249), and the positive rate of mNGS was 79.6% (82/103). A total of 28 kinds of pathogenic microorganisms were cultured from conventional culture, and 58 kinds of pathogenic microorganisms were detected by mNGS, including *Mycobacterium*, *Rickettsia*, and *Chlamydia psittaci*. In conventional culture, the most frequent Gram-negative bacteria, Gram-positive bacteria, and fungi were *Klebsiella pneumoniae*, *Corynebacterium striatum*, and *Candida glabrata*, and those with the highest frequency of occurrence in mNGS detection were *Acinetobacter baumannii*, *Enterococcus faecium*, and *Aspergillus flavus*.

Conclusions: Throughout the whole treatment process, different kinds of suspicious biological specimens of high-infection-risk ICU patients supported by ECMO should undergo both mNGS detection and traditional culture early and repeatedly.

KEYWORDS

metagenomic next-generation sequencing, conventional culture, extracorporeal membrane oxygenation, non-organ transplant, different therapy periods

Introduction

Extracorporeal membrane oxygenation (ECMO), also known as extracorporeal life support, is an important mechanism for the treatment of critical illness. It is typically divided into veno-venous ECMO (VV ECMO) and veno-arterial ECMO (VA ECMO) based on the types of blood vessels drained and reinfused and the types of organs supported (Professional Committee for Extracorporeal Life Support of Chinese Medical Doctor Association, 2018). ECMO acts as a bridge between critical illnesses (e.g., cardiogenic shock, septic shock, and respiratory failure) and further treatment or decision. Moreover, because it can quickly and effectively replace vital organ functions, ECMO is considered a last resort to save lives in emergency rescue (Ventetuolo and Muratore, 2014; Guglin et al., 2019). Nowadays, ECMO is widely used in the intensive care unit (ICU). Timely and correct application of ECMO can effectively improve the survival rate of severe patients and even improve the prognosis (Oh et al., 2021; Kochanek et al., 2022). However, the infection rate of severe patients should not be underestimated. An invasive procedure is one of the important risk factors of infection in severe patients, including continuous renal replacement therapy (CRRT), intra-aortic balloon pump counterpulsation (IABP), deep venipuncture, tracheal intubation, and even ECMO cannulas (Kühn et al., 2020). Infections are common complications in ECMO patients and are associated with an increase in mortality (Burket et al., 1999). Early and accurate identification of pathogenic microorganisms is the key to the treatment of infection.

Traditional bacterial culture has been used in clinical practice for many years, which can cultivate pathogenic bacteria and conduct drug sensitivity tests. However, ECMO patients often suffer from mixed infections and special pathogen infections due to long hospital stays and physical weakness. At this time, a traditional bacterial culture is limited. Metagenomic next-generation sequencing (mNGS) technology is widely used to explore the pathogens implicated in various infectious diseases by testing the DNA of all microorganisms directly extracted from clinical specimens (Zhang et al., 2020; Yue et al., 2021). The advantages of mNGS detection are high efficiency and accuracy and being a non-culture-based method. Recently, mNGS has been applied to pathogen detection in infectious diseases as a universal method to sequence and identify nucleic acids from microorganisms. In addition to high efficiency and accuracy, the advantages of mNGS include being a non-culture-based method.

At present, few literature comprehensively and detailedly analyzes the detection of pathogens using both mNGS and traditional culture in ECMO patients at different hospitalization and treatment periods. In the present study, we analyzed the data from 62 non-organ transplant critically ill patients supported by ECMO in the First Affiliated Hospital of Zhengzhou University.

Materials and methods

Patients and study design

The present study retrospectively enrolled ECMO-assisted patients who were detected to have pathogenic microorganisms by both mNGS and traditional culture from October 2020 to October 2022 at the First Affiliated Hospital of Zhengzhou University, respectively. The inclusion criteria were as follows: 1) the necessity to use ECMO, 2) patients had completed ECMO treatment (more than 48 h), and 3) detected pathogenic microorganisms by both mNGS and traditional culture at least once. The exclusion criteria were as follows: 1) aged <18 years, 2) pregnant, and 3) ECMO used to assist organ transplantation operation. During this period, 152 patients who had indications for ECMO assistance were detected to have pathogenic microorganisms by both mNGS and traditional culture. Among them, 84 organ transplantation patients and six patients with ECMO assistant time less than 48 h were excluded. Finally, 62 patients, including 46 men and 16 women, were enrolled and divided into the non-survivor group (n = 38) and the survivor group (n = 24) according to their discharged status. Then, based on the type of ECMO assistance, patients were divided into the VV ECMO group (n = 43) and the VA ECMO group (n = 19).

The baseline characteristics, clinical data, and pathogens of 62 critically ill patients assisted by ECMO were recorded and analyzed. The clinical data of the ECMO patients were obtained through the electronic medical record system. We used Sequential Organ Failure Assessment (SOFA), Acute Physiology and Chronic Health Evaluation II (APACHE II) scores, and the D-values of SOFA and APACHE II scores to evaluate the patients' condition. The D-values of SOFA and APACHE II scores were defined as the highest score of 48 h of ECMO assistance subtracted from the highest score before ECMO assistance.

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University, Zhengzhou,

China (no. 2020-KY-429). All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki. All participants provided written informed consent before participation.

ECMO application

ECMO equipment adopts an ECMO system kit, including a centrifugal pump, oxygenator, and its connecting pipeline, among which the centrifugal pump adopts Rotaflow pump head and controller from Medtronic and MAQUET. The oxygenator is divided into Medtronic and MAQUET adult oxygenators. The pipeline includes an ECMO kit pipeline and an ordinary PVC pipeline, and the cannulas are all arteriovenous thin-walled cannulas. The commonly used site for patients assisted by VV ECMO is the femoral vein–internal jugular vein, and the puncture method was percutaneous venous catheterization. In VA ECMO patients, sites of vascular access were the femoral vessels.

VV ECMO management 1) Management of mechanical ventilation: to avoid or reduce the occurrence of ventilator-related lung injury to the greatest extent while promoting the recruitment of collapsed alveoli. 2) Anticoagulation: unfractionated heparin anticoagulation to maintain activated coagulation time (ACT) is 1.5 times the upper limit of normal, and the activated partial thromboplastin time (APTT) is 40–55 s. 3) Analgesia and sedation: adequate analgesia, sedation combined with muscle relaxants, combined with the patients' human–machine synchronization, and the complete suppression of spontaneous breathing. 4) Flow management: adjust the flow according to the patients' monitoring indicators to maintain blood oxygen saturation (SaO_2) at 85%–95% and PaO_2 above 60 mmHg; VV ECMO pump flow to 2–3 L/min, close VV ECMO airflow, under certain respiratory support (FiO_2 < 50%, positive end-expiratory pressure (PEEP) ventilation ≤10 cmH_2O, and peak airway pressure <30 cmH_2O), monitor for 2–4 h, and if SaO_2 > 95% and $PaCO_2$ < 50 mmHg, weaning of VV ECMO can be considered.

In VA ECMO-assisted patients, the prompt number of revolutions per minute of the ECMO device was adjusted to meet the standard that the patients' cardiac index of greater than 2.2 L/min/m^2, central mixed venous oxygen saturation above 70%, and the mean arterial pressure above 65 mmHg. Usually, an arterial catheter was used to continuously monitor blood pressure. Weaning of VA ECMO can be considered if a flow of 0.5–1.0 L/min is achieved. Every stage of reductions in blood flow must be accessed by echocardiographic and hemodynamic assessments.

Methods and process of mNGS

The samples were collected from the ECMO patients based on clinically therapeutic requirements. About 5 ml of sample was used to extract DNA by TIANamp Micro DNA Kit following the manufacturer's instructions. DNA libraries were constructed by DNA fragmentation, end-repair, adapter ligation, and PCR amplification, followed by sequencing. Agilent 2100 Bioanalyzer (Agilent, USA) and ABI StepOnePlus Realtime PCR System were used for quality control of the DNA library; the qualified libraries were sequenced on NextSeq 550Dx platform (Illumina, USA) using 75-bp sequencing read length.

Illumina NextSeq 550 sequencer was used for the metagenomics sequence, and 15–20 samples that contain one negative control were loaded in each metagenomics sequencing batch. The internal reference, which is from *Arabidopsis thaliana*, was provided by sequencing manufacturers. High-quality sequencing data were generated by removing low-quality and short reads (<35 bp in length) and then yielding reads strictly aligned to pathogen species (SDSMRN) and reads strictly aligned to pathogen genus (SDSMRNG). The list of microorganisms obtained through the above analysis process was compared with an internal background database containing microorganisms present in more than 50% of samples in the laboratory over the past 3 months. Suspected background microorganisms were removed. Microorganisms with SDSMRN > 50 and at least three times higher than the control group were considered suspected pathogens, while the SDSMRN of suspected pathogens with SDSMRN < 50 should be at least five times higher than that of the control group.

The data that support the findings of this study have been deposited into the EMBL database with accession number PRJEB57970.

Statistical analysis

All collected data were statistically analyzed using SPSS 21.0 (IBM Corp., Armonk, NY, USA). Variables of the two groups were compared using the Wilcoxon rank-sum test for continuous variables and the chi-square test for binary and categorical variables, as appropriate. $p < 0.05$ indicates that the difference is statistically significant.

Results

Characteristics of ECMO patients

The flowchart of this experiment is shown in Figure 1. As shown in Table 1, the survival ECMO patients were younger than the non-survivors (60 ± 14 *vs.* 49 ± 17 years, p = 0.008, Table 1). In general, the primary diseases of ECMO patients who underwent detection by mNGS and traditional culture were infectious shock (46.8%), respiratory failure (35.5%), and cardiogenic shock (11.3%). Before ECMO assistance, there was no significant difference between the survivors and non-survivors in the improvement of the condition evaluated by the two scoring systems (SOFA and APACHE II). Similarly, there was no significant difference between the two groups after 48 h of ECMO assistance in the improvement of the condition evaluated by the SOFA scores. However, the non-survivor group scored higher than the survivor group as evaluated by the APACHE II scores after 48 h of ECMO assistance (23.42 ±

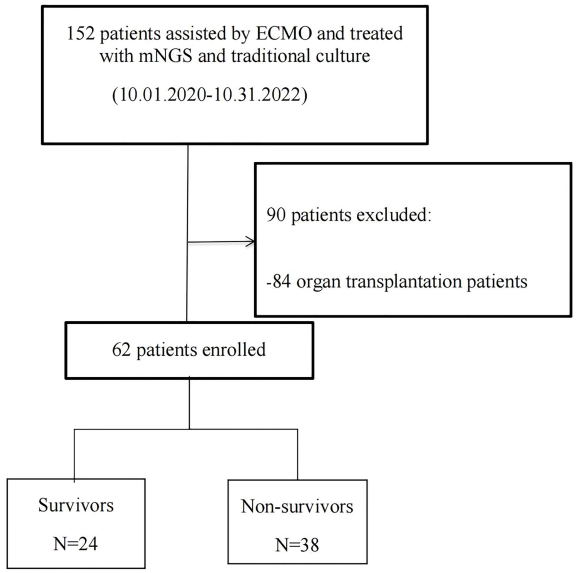

FIGURE 1
Flowchart of analyzed patients. ECMO, extracorporeal membrane oxygenation; mNGS, metagenomic next-generation sequencing.

7.277 *vs.* 18.58 ± 6.520, p = 0.010) (Table 1). Within 48 h of ECMO support, there was no significant difference between the two groups in the improvement of the condition as evaluated by the two scoring systems (SOFA and APACHE II) (Table 1). Among the enrolled patients, the support type of VA ECMO (30.6%) was significantly less than that of VV ECMO (69.4%), but there was no significant difference between the survivors and non-survivors. In addition, in terms of invasive operations related to infection, there was no statistical difference between the two groups in CRRT use rate, IABP use rate, ECMO support time, and mechanical ventilation time (Table 1).

Laboratory examinations of ECMO patients

There was no significant difference in the liver (alanine transaminase (ALT) and aspartate transaminase (AST)) and kidney function (creatinine (Cr)), coagulation function (prothrombin time (PT) and APTT), and heart function (N-terminal pro-B-type natriuretic peptide (NT-pro-BNP)) between the non-survivor group and the survivor group within 48 h after ECMO support or on admission (Tables 2, 3). Compared with

TABLE 1 Baseline characteristics of ECMO patients.

Variables	Non-survivors (n = 38)	Survivors (n = 24)	p-Value
Male (n%)	31 (81.6)	15 (62.5)	0.118
Age* (years)	60 ± 14	49 ± 17	0.008
BMI (kg/m^2)	23.8 ± 2.6	25.5 ± 9.2	0.304
Comorbidities (n%)			
Hypertension	7 (18.4)	10 (41.7)	0.078
Type 2 diabetes	8 (21.1)	4 (16.7)	0.670
Coronary heart disease	6 (15.8)	3 (12.5)	0.720
Primary disease (n%)			0.088
Septic shock	20 (52.6)	9 (37.5)	
Respiratory failure	15 (39.5)	7 (29.2)	
Cardiogenic shock	2 (5.3)	5 (20.8)	
Others	1 (2.6)	3 (12.5)	
CRRT (n%)	17 (44.7)	12 (50)	0.692
IABP (n%)	1 (2.6)	3 (12.5)	0.191
Baseline SOFA and APACHE II scores			
SOFA scores (before ECMO assistance)	9.23 ± 4.224	9.82 ± 4.231	0.648
SOFA scores (48 h of ECMO assistance)	11.97 ± 4.290	11.67 ± 4.622	0.791
APACHE II scores (before ECMO assistance)	21.43 ± 8.190	22.18 ± 9.098	0.775
APACHE II scores (48 h of ECMO assistance)	23.42 ± 7.277	18.58 ± 6.520	0.010
SOFA D-value	2 (0–4)	0 (–2–3)	0.102
APACHE II D-value	0 (–5–7)	0 (–12–1)	0.147

(Continued)

TABLE 1 Continued

Variables	Non-survivors (n = 38)	Survivors (n = 24)	p-Value
ECMO type			0.721
VV ECMO	27 (71.1)	16 (66.7)	
VA ECMO	11 (28.9)	8 (33.3)	
ECMO assistance **time** (h)	203 (123–304)	133 (87–189)	0.053
Mechanical ventilation time (h)	293 (219–444)	220 (145–443)	0.368

The data are shown as the mean ± SD, median (interquartile 25-75), or n (percentage).
ECMO, extracorporeal membrane oxygenation; BMI, body mass index; CRRT, continuous renal replacement therapy; IABP, intra-aortic balloon pump; SOFA, Sequential Organ Failure Assessment; APACHE, Acute Physiology and Chronic Health Evaluation.
*Significant difference ($p < 0.05$).

baseline levels of lactate, the data showed an increase in lactate levels within 48 h after ECMO assistance. In terms of inflammation indicators, we selected blood leukocyte, procalcitonin, C-reactive protein, interleukin (Mandawat and Rao, 2017; Kühn et al., 2020), interferon-γ, and $CD4^+$ T-cell absolute count for comparison, but no statistical difference was observed between the two groups during the two time periods (Tables 2, 3).

Samples of mNGS and traditional culture

Of the 62 enrolled ECMO patients, 103 samples were submitted for mNGS detection, including 37 (35.9%) blood samples and 56 (54.4%) bronchoalveolar lavage fluid (BALF) samples. Of the above samples, 82 (79.6%) were positive. However, 1,249 samples were successively sent for traditional culture, of which 380 (30.4%) were positive. Although the number of samples submitted for examination by traditional culture was more than that for mNGS detection, the types of pathogenic microorganisms cultured were less than those detected by mNGS (28 *vs.* 58). The spectrum of pathogenic microorganisms detected by the two methods was not identical because only 17 pathogenic microorganisms can be detected by both detection methods (Figure 2). Traditional culture cannot cultivate viruses and special microorganisms (such as *Rickettsia* and *Chlamydia*) that can be detected by mNGS (Figure 3).

TABLE 2 Baseline laboratory examinations of ECMO patients.

Laboratory examinations	Non-survivors (n = 38)	Survivors (n = 24)	p-Value
WBC (10^9/L)	14.8 ± 7.4	12.5 ± 7.4	0.310
Hb (g/L)	119.1 ± 24.4	117.1 ± 24.6	0.798
PLT (10^9/L)	160.3 ± 91.1	168 ± 75.6	0.770
ALT (U/L)	32 (21–61)	49 (11–176)	0.312
AST (U/L)	38 (27–118)	95 (64–125)	0.311
Cr (μmol/L)	66 (57–121)	95 (64–135)	0.740
PT (s)	13.7 ± 2	13.6 ± 3.8	0.875
APTT (s)	33.4 ± 15.2	41.4 ± 33.7	0.275
NT-pro-BNP (pg/ml)	2,170 (506–4,960)	178 (78–3,227)	0.627
Lactate (mmol/L)	2 (1.4–4.7)	2.5 (1.5–5.6)	0.812
PCT (ng/ml)	0.4 (0.2–3)	1.5 (0.2–29.6)	0.273
CRP (mg/L)	123.3 (53.5–205.3)	54.3 (11.61–171.6)	0.871
IL-6 (pg/ml)	103.7 (45.9–217)	64.9 (10.8–80.5)	0.075
IL-10 (pg/ml)	8 (6–16.5)	12.8 (5.2–27.8)	0.577
IFN-γ (pg/ml)	2.1 (1–3.2)	2 (1–9.1)	0.622
$CD4^+$ T-cell absolute count (/μl)	142.7 (59.5–241.7)	256.8 (70.9–556.1)	0.866

The data are shown as the mean ± SD or median (interquartile 25-75).
ECMO, extracorporeal membrane oxygenation; WBC, white blood cell; Hb, hemoglobin; PLT, platelet; ALT, alanine transaminase; AST, aspartate transaminase; Cr, creatinine; PT, prothrombin time; APTT, activated partial thromboplastin time; PCT, procalcitonin; CRP, C-reactive protein; IL, interleukin; IFN, interferon.

10.3389/fcimb.2023.1146088

TABLE 3 Laboratory examinations of ECMO patients within 48 h after ECMO assistance.

Laboratory examinations	Non-survivors (n = 38)	Survivors (n = 24)	p-Value
WBC (10^9/L)	12.4 ± 7.1	11.7 ± 6.8	0.679
Hb (g/L)	93.6 ± 17.1	94.8 ± 21	0.809
PLT (10^9/L)	104.7 ± 75.2	121.4 ± 87.4	0.427
ALT (U/L)	29 (18–130)	54 (31–169)	0.371
AST (U/L)	51 (29–156)	94 (39–308)	0.420
Cr (μmol/L)	70 (57–132)	105 (76–174)	0.356
PT (s)	16.1 ± 3.5	16.6 ± 4.4	0.577
APTT (s)	49.5 ± 31.4	55.2 ± 35	0.512
NT-pro-BNP (pg/ml)	1,710 (587–6,279)	2,350 (277–4,871)	0.948
Lactate (mmol/L)	2.9 (2.1–4.5)	4.6 (2–8.2)	0.131
PCT (ng/ml)	3.8 (1.2–14.1)	3.3 (0.8–31.1)	0.629
CRP (mg/L)	150 (70.4–213.7)	93.5 (39.5–191.2)	0.087
IL-6 (pg/ml)	77.1 (7.8–464.2)	42.5 (13–443.8)	0.810
IL-10 (pg/ml)	4.3 (2.8–18.8)	18.4 (7.7–63.5)	0.893
IFN-γ (pg/ml)	1.4 (0.6–3.9)	1.5 (1.2–2.2)	0.464
$CD4^+$T cell absolute count (/μl)	s233.7 (84.6–426.1)	135.4 (102.1–454.2)	0.615

The data are shown as the mean ± SD or median (interquartile 25-75).
ECMO, extracorporeal membrane oxygenation; WBC, white blood cell; Hb, hemoglobin; PLT, platelet; ALT, alanine transaminase; AST, aspartate transaminase; Cr, creatinine; PT, prothrombin time; APTT, activated partial thromboplastin time; PCT, procalcitonin; CRP, C-reactive protein; IL, interleukin; IFN, interferon.

Pathogenic microorganisms in different treatment periods

In conventional culture, the most frequent Gram-negative bacteria, Gram-positive bacteria, and fungi were *Klebsiella pneumoniae*, *Corynebacterium striatum*, and *Candida glabrata*. Those with the highest frequency of occurrence of mNGS detection were *Acinetobacter baumannii*, *Enterococcus faecium*, and *Aspergillus flavus* (Figure 3). Moreover, the human herpes virus was the most prevalent virus detected by mNGS in ECMO patients (Figure 3). Figure 4 shows the details of pathogenic microorganisms in non-survivors and survivors of different ECMO assistance types (VV ECMO and VA ECMO) in different time periods (before ECMO assistance, within 48 h of ECMO assistance, after 48 h of ECMO assistance, and after weaning of ECMO). It can be seen from Figure 4 that the mNGS detection technology detected pathogenic microorganisms earlier than traditional culture in VV ECMO-assisted patients. In addition, mNGS can help diagnose special infections, including mixed infections and infections caused by special pathogens. In Figure 5, to avoid some patients assisted by ECMO only in the late stage of treatment, the time period has been re-divided into three different times: 48 h after admission, between 48 h and 7 days of admission, and after 7 days of admission. Combining Figures 4, 5, it can be seen that patients supported by VA ECMO usually undergo detection of pathogenic microorganisms later. In addition, although the sensitivity of mNGS was high, the times of traditional culture were significantly more than those of mNGS detection.

Discussion

In this single-center study involving non-organ transplantation critically ill patients assisted with ECMO, we once again verified that the effectiveness and comprehensiveness of mNGS detection are higher than those of traditional culture, and we found that the traditional culture and mNGS detection are inconsistent. More importantly, we found that different types of ECMO and different treatment periods have different pathogenic microorganisms and different clinical tendencies of detection methods.

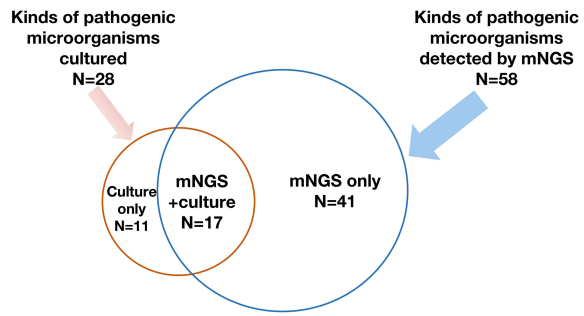

FIGURE 2
A total of 58 kinds of pathogenic microorganisms were detected by mNGS, while 28 of them were cultured from conventional culture, and 17 kinds of pathogenic microorganisms were found by both mNGS and conventional culture. mNGS, metagenomic next-generation sequencing.

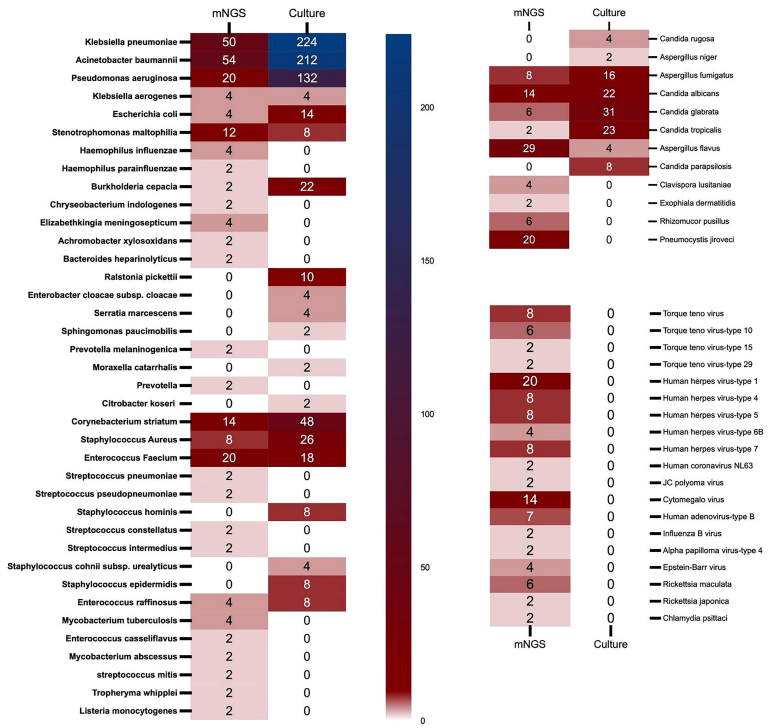

FIGURE 3
The heat map of pathogenic microorganisms details in different methods (mNGS and conventional culture). mNGS, metagenomic next-generation sequencing.

ECMO is a percutaneously implantable mechanical circulatory assisting technology, which has the advantages of convenient insertion independent of location and simultaneous dual ventricular combined breathing assistance. Therefore, in recent years, it has been applied in cases of acute circulatory and/or respiratory failure where conventional life support is ineffective (Mandawat and Rao, 2017). The operations of ECMO preparation, boarding, and weaning have all been rapidly improved in practice, and the survival rate of critically ill patients has been greatly improved as a result. However, the mortality rate of ECMO patients is still very high, and the reasons are considered to be closely related to the patients' primary disease, complications, infection, and other factors. Infections could increase the morbidity and mortality of hospitalized critically ill patients. The incidence of infections in ECMO patients reported in the literature varies widely, fluctuating between 20% and 60% (Bizzarro et al., 2011; Schmidt et al., 2012; Austin et al., 2017; Grasselli et al., 2017). All ECMO patients included in this article had definite infections during hospitalization. In clinical work, the cultivation and detection of pathogenic microorganisms are often carried out with the change of the disease, which is different from well-planned clinical trials. Therefore, mNGS detection and traditional culture in the real world may provide a more realistic reference and help guide the clinical application of various pathogen detection methods.

Our research results found that in conventional culture, the most frequent Gram-negative bacteria, Gram-positive bacteria, and fungi were *K. pneumoniae*, *C. striatum*, and *C. glabrata*. Those with the highest frequency of occurrence of mNGS detection were *A. baumannii*, *E. faecium*, and *A. flavus*. Of course, this conclusion would be interfered with to some extent because traditional culture is inexpensive and usually repeated using different samples. It can also be seen from this article that there were 103 samples of mNGS and 1,249 samples of traditional culture. The positive rate of traditional culture (30.4%) was lower than that of mNGS detection (79.6%), which was consistent with previous studies (Cheng et al., 2019). Then, we found that mNGS detected various viruses, *Rickettsia*, *Chlamydia*, *Mycobacterium*, *Tropheryma whipplei*, and *Listeria monocytogenes*. These microorganisms were clinically confirmed as pathogens, which again confirmed the necessity of mNGS detection in special pathogen infection and mixed infection. Then, after dividing the patients in detail according to the length of stay, we found that some of the results of mNGS detection were earlier than those of the traditional culture, and the infection of VV ECMO patients was significantly earlier than that of VA ECMO patients, which may be due to the infection of VV ECMO patients before the application of ECMO, and the infection rate of arterial ECMO cannulas was lower than that of venous ECMO cannulas.

The technology of mNGS has already been widely used in clinical practice. For the diagnosis of infectious diseases, mNGS is highly sensitive, rapid, and widely available (Brown et al., 2018; Carpenter et al., 2019). In addition, mNGS does not require the isolation of pathogenic bacteria and is not affected by the use of antibiotics, thus reducing the false-negative rate. Notably, mNGS

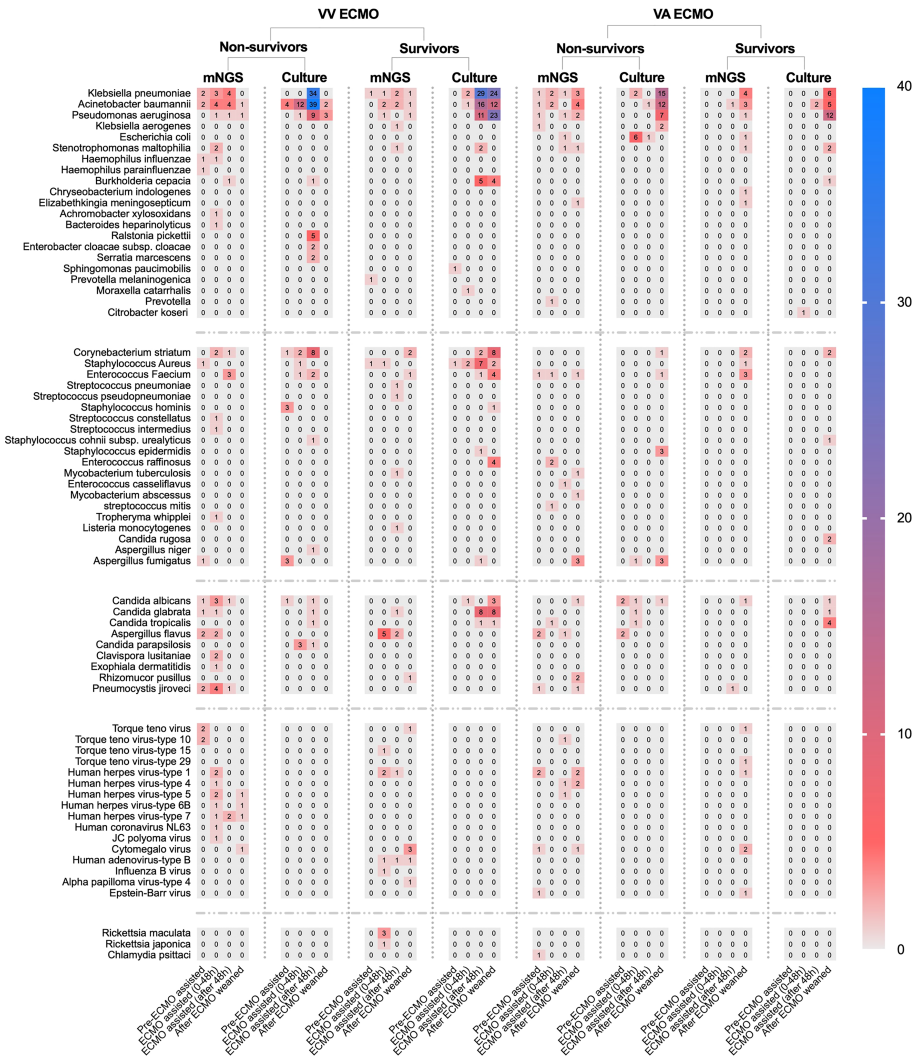

FIGURE 4
The heat map of pathogenic microorganism details in non-survivors and survivors of different ECMO assistance types (VV ECMO and VA ECMO) in different time periods (before ECMO assistance, within 48 h of ECMO assistance, after 48 h of ECMO assistance, and after weaning of ECMO). ECMO, extracorporeal membrane oxygenation; VV, veno-venous; VA, veno-arterial.

assay can theoretically detect all the nucleotide sequences present within a given sample, allowing for the simultaneous detection of multiple pathogens. A recent study found that mNGS samples could provide more accurate diagnostic information for infections (Chen et al., 2021). In the case of concomitant prophylactic antibiotic treatment, early mNGS may accurately guide treatment regimens. In patients whose causative organism remains elusive, we envisage that the incidence of infections will be lowered by reducing the duration of prophylactic antibiotic application. Meanwhile, patients in whom the suspected causative organism has been detected may benefit from a more precise and individualized form of treatment. Thus, mNGS is likely to prevent the development of infections caused by antibiotic abuse. There are many advantages of mNGS detection. However, traditional training is still indispensable. In our single-center study, 39.3% of the pathogens in traditional culture were not detected by mNGS. The combination of traditional culture and mNGS detection to find pathogens and conduct drug sensitivity tests is more helpful to guide clinical treatment in the real world.

This study was a single-center retrospective study with a small sample size. Thus, a large, multi-center, prospective randomized controlled study is needed to further verify the results.

Conclusion

During the treatment of severe patients supported by ECMO, there were different kinds of pathogenic microorganisms infected in

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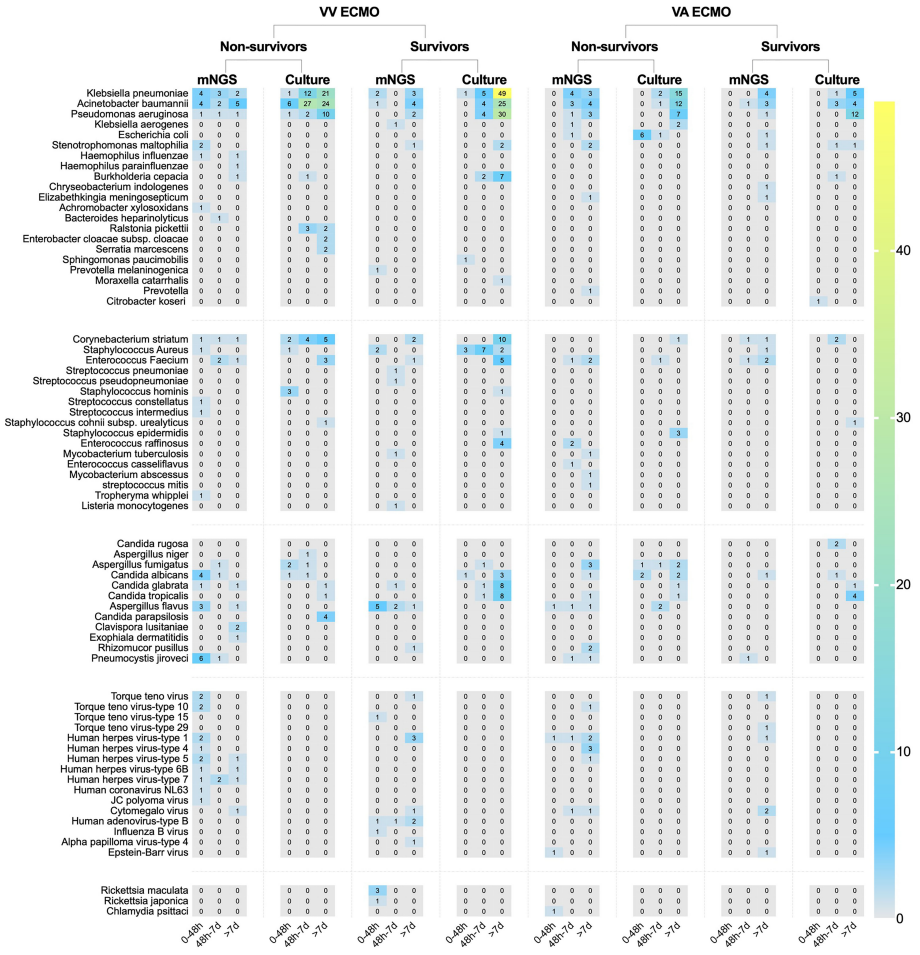

FIGURE 5
The heat map of pathogenic microorganism details in non-survivors and survivors of different ECMO assistance types (VV ECMO and VA ECMO) in different time periods (within 48 h after admission, between 48 h and 7 days of admission, and after 7 days of admission). ECMO, extracorporeal membrane oxygenation; VV, veno-venous; VA, veno-arterial.

different treatment stages and different types of ECMO. The combination of multiple applications of mNGS detection and traditional culture could improve the detection rate of pathogenic microorganisms.

Data availability statement

The data that support the findings of this study have been deposited into EMBL database with accession number PRJEB57970.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XZ and L-PB: software, formal analysis, data curation, and writing—original draft. XZ, Y-CZ and B-YL: conceptualization, methodology, and writing—review and editing. Y-CZ and Z-ZY: validation and formal analysis. X-YZ: term, resources, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

Funding

This study was partly supported by the Joint Project of Medical Science and Technology of Henan (LHGJ20190095) awarded to Dr.

XZ, MD, and the Program for Science and Technology Development of Henan Province (212102310210) awarded to Pro. X-YZ, MD, PhD.

Acknowledgments

The authors would like to acknowledge all the patients and health staff who participated in this study.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1146088/full#supplementary-material

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frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 10 November 2023
DOI 10.3389/fcimb.2023.1107990

OPEN ACCESS

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RECEIVED 25 November 2022
ACCEPTED 03 March 2023
PUBLISHED 10 November 2023

CITATION
Sun X, Song J, Leng X, Li F, Wang H, He J, Zhai W, Wang Z, Wu Q, Li Z and Ruan X (2023) A preliminary evaluation of targeted nanopore sequencing technology for the detection of *Mycobacterium tuberculosis* in bronchoalveolar lavage fluid specimens. *Front. Cell. Infect. Microbiol.* 13:1107990. doi: 10.3389/fcimb.2023.1107990

A preliminary evaluation of targeted nanopore sequencing technology for the detection of *Mycobacterium tuberculosis* in bronchoalveolar lavage fluid specimens

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Objective: To evaluate the efficacy of targeted nanopore sequencing technology for the detection of *Mycobacterium tuberculosis*(*M.tb.*) in bronchoalveolar lavage fluid(BALF) specimens.

Methods: A prospective study was used to select 58 patients with suspected pulmonary tuberculosis(PTB) at Henan Chest Hospital from January to October 2022 for bronchoscopy, and BALF specimens were subjected to acid-fast bacilli (AFB) smear, *Mycobacterium tuberculosis* MGIT960 liquid culture, Gene Xpert MTB/RIF (Xpert MTB/RIF) and targeted nanopore sequencing (TNS) for the detection of *M.tb.*, comparing the differences in the positive rates of the four methods for the detection of patients with different classifications.

Results: Among 58 patients with suspected pulmonary tuberculosis, there were 48 patients with a final diagnosis of pulmonary tuberculosis. Using the clinical composite diagnosis as the reference gold standard, the sensitivity of AFB smear were 27.1% (95% CI: 15.3-41.8); for *M.tb* culture were 39.6% (95% CI: 25.8-54.7); for Xpert MTB/RIF were 56.2% (95% CI: 41.2-70.5); for TNS were 89.6% (95% CI: 77.3-96.5). Using BALF specimens Xpert MTB/RIF and/or *M.tb.* culture as the reference standard, TNS showed 100% (30/30) sensitivity. The sensitivity of NGS for pulmonary tuberculosis diagnosis was significantly higher than Xpert MTB/RIF, *M.tb.* culture, and AFB smear. Besides, P values of <0.05 were considered statistically significant.

Conclusion: Using a clinical composite reference standard as a reference gold standard, TNS has the highest sensitivity and consistency with clinical diagnosis, and can rapidly and efficiently detect PTB in BALF specimens, which can aid to improve the early diagnosis of suspected tuberculosis patients.

KEYWORDS

targeted nanopore sequencing, *Mycobacterium tuberculosis*, bronchoalveolar lavage fluid, detection, pulmonary tuberculosis

Introduction

Tuberculosis (TB) is an infectious disease caused by *M.tb.* and poses a major threat to human health. According to the World Health Organization (WHO) Global TB annual report in 2021, China estimates that there are about 780,000 TB cases and the estimated TB incidence rate is about 55 cases per 100,000 people. China ranks 3rd in estimated TB incidence among 30 high TB burden countries, below Indonesia and India. Pathogenic positive rate of TB in China is only 58% (World Health O, 2022). Therefore, rapid and accurate clinical diagnostic methods are essential to improve the diagnosis of tuberculosis, treat patients with tuberculosis, and prevent the spread of tuberculosis.

Nowadays, in the general testing clinical diagnosis method of *M.tb.*, that AFB smear has quick and easy test method, low cost advantage, but pathogen positivity rate is low, *M.tb.* incubation takes a long time, which cannot applicable to clinical rapid diagnosis. *M.tb.* culture-based phenotypic drug susceptibility testing (DST) is impacted by biosafety, long incubation time and the decreased incubation rate after application of anti-tuberculosis drugs. The slow-growing feature of Mycobacterium tuberculosis makes this methodology take a long time and uncertainty in results due to possible poor cultivation or microbial contamination which cannot fulfill the requirements of rapid clinical diagnosis. The rapid and accurately testing specimens for *M.tb.* and the rapid diagnosis of drug-resistant tuberculosis are essential for the effective control of drug-resistant tuberculosis epidemic. The drug-resistant phenotype in *M.tb.* is confirmed primarily by chromosomal mutations in several genes (Gygli et al., 2017). For example, rifampicin resistance caused by mutations in the *rpoB* gene encoding the β subunit of RNA polymerase is the most common gene mutation in *M.tb.* Xpert MTB/RIF assays for *M.tb.* and *rpoB* gene mutations directly from sputum using semi-nested PCR. Xpert MTB/RIF was initially recommended by WHO for TB diagnosis in 2010, however, Xpert MTB/RIF showed less sensitive test results for samples with low bacterial load. Moreover, the assay only detects rifampicin resistance mutations and cannot report the type of mutation.

With the rapidly development of Next-generation sequencing (NGS) in recent years, WHO recommends NGS for detection of drug resistance related mutations in the Mycobacterium tuberculosis complex(MTBC) and targeted high-throughput sequencing technology enables rapid detection of both common and rare genetic variations. Targeted NGS is focused on sequencing a select set of genes or gene regions that have known or suspected associations with a specific pathogen (e.g., *M.tb.*) or a specific phenotype (e.g. drug resistance)(World Health O, 2018). The method has the advantages of low sequencing cost, easy customization, high-throughput, rapid testing and rapid reporting of test results, and does not dependent on the *M.tb.* culture.

In this study, the performance of TNS in BALF specimens was prospectively evaluated, and the clinical diagnostic value for *M.tb.* was evaluated. This study also compared the differences between TNS and AFB smear, Xpert MTB/RIF, and *M.tb.* culture in the clinical diagnostic effectiveness of tuberculosis.

Materials and methods

Ethical approval

This study was approved by the Ethics Committee of Henan Provincial Chest Hospital, No. (2021) Ethics No. (11-04). Written informed consent was signed by all participants in this study.

Patient recruitment

Patients with clinical diagnosis of pulmonary tuberculosis were enrolled prospectively and consecutively at Henan Chest Hospital from January to October 2022. Clinical diagnostic criteria for tuberculosis refer to the *WS196-2017 Classification of Tuberculosis* (China health industry, 2017) and *WS288-2017 Diagnostic Criteria for Tuberculosis* (Xia et al., 2018). Patients had undergone all routine clinical examinations before enrollment, including: chest CT, sputum smear for molecular assay, tuberculin skin test (TST), and *M.tb.* enzyme-linked immunospot (ELISPOT) assay. 10 patients with non-pulmonary tuberculosis included 4 cases of pulmonary Non-tuberculous mycobacteriosis, 3 cases of pneumomycosis, 1 case of pneumonia, 1 case of pulmonary sequestration and 1 case of nocardiosis. The diagnosis of the above patient was confirmed by bacteriological culture of the BALF, genetic testing of the BALF and postoperative pathological results.

Inclusion and exclusion criteria

Inclusion criteria

Being aged 15–80 years; clinical symptoms related to pulmonary tuberculosis (e.g., cough, fever, hemoptysis); abnormal imaging of pulmonary tuberculosis; positive ELISPOT test or positive TST; patients have informed the purpose of the study and signed an informed consent form.

Exclusion criteria

Patient refusal to perform an invasive examination; bleeding tendency or coagulation disorders; severe cardiorespiratory dysfunction; The size of the specimens sent for testing cannot fulfill the testing requirements.

Specimen collection

Patients were included in the bronchoscopy, and bronchoalveolar lavage was performed on the corresponding lesions in combination with the patient's chest CT imaging performance: Saline was instilled into the bronchopulmonary segment of the lesion, and the BALF was recovered by negative pressure suction and placed in a sterile bottle, which could be repeated until 20mL of BALF was collected; the collected BALF was placed equally in tubes A, B, C, and D and sealed, and tube A was sent for antacid staining smear, tube B for tuberculosis culture, tube C for Xpert MTB/RIF assay, and tube D for TNS.

Main equipment and test methods

AFB smear

N-acetyl-l-cysteine–NaOH was used for digestion and decontamination of the specimens, and auramine O, a fluorescentdye, was used for direct smear microscopy and confirmed by staining with Ziehl–Neelsen, based on the WHO guidelines.

Mycobacterium tuberculosis culture and phenotypic DST

N-acetyl-l-cysteine–NaOH for digestion and decontamination of specimens, Liquid medium (Bactec MGIT 960 Mycobacterial Culture System) was used for Mycobacterium tuberculosis culture. All MTB isolates were validated by both a growth test on p-nitrobenzoic acid containing medium and an MBP 64 antigen detection kit. Non-tuberculosis mycobacteria (NTM) were excluded. Antimicrobial susceptibility testing (AST) use MicroDST^{TM} test kit. All steps were performed by trained and specialized staff in a biosafety cabinet in accordance with the relevant guidelines. The critical concentrations were 0.2μg/mL for isoniazid, 2.0μg/mL for streptomycin, 5.0μg/mL for ethambutol, 1.0μg/mL for rifampin, 2.0μg/mL for ofloxacin, 0.5μg/mL for moxifloxacin,2.0μg/mL for capreomycin, 1.0μg/mL for amikacin, 2.5μg/mL for prothionamide, 2.0μg/mL for aminosalicylic acid, respectively. Pyrazinamide phenotypic DST was performed using the Middlebrook 7H10 agar medium. The critical concentration was 100.0μg/mL for pyrazinamide. The liquid culture-positive strains were extracted, adjusted to the specified concentrations, then inoculated into drug-containing medium and control medium to be cultured at 36°C ± 1°C for 14 days, drug susceptibility was determined by the number of colonies grown on the drug-containing medium and the number of colonies grown on the control medium(Baso, Zhuhai, China).

Xpert MTB/RIF

Mix the centrifuged lavage solution specimen with 2ml of sample processing solution. After stirring for 20 seconds, the mixed solution was incubated at room temperature for 15 minutes. Then, the first generation Xpert MTB/RIF reaction cassette is added to the 2ml of the mixed solution; the cassette is then placed into the test module for automated testing. The system automatically reads the MTB test results within 2 hours(Cepheid, Sunnyvale, CA, USA).

Targeted nanopore sequencing

200μL sample was taken into an EP tube and an equal volume of DTT solution, 10μL of proteinase K, 5μL of lysozyme, and 0.05mm Zirconium oxide grinding beads were added, and ground using a grinder. The supernatant was used for nucleic acid extraction by using QIAamp DNA Microbiome Kit (Qiagen). The concentration of extracted DNA was determined by dsDNA Assay Kit (ThermoFisher). The extracted DNA was amplified by multiplex PCR. The PCR product was purified by magnetic beads, and the eluted product was taken for qubit quantitative quality control. Equal quality PCR products with different barcode labels were pooled, and the pooled library was followed by the nanopore ligation kit. Nanopore sequencing and data analysis: 1) 100ng of the final prepared library was taken for up-sequencing; 2) the GridION platform was used for sequencing and MinKNOW software was used to collect real-time sequencing data; 3) the original sequencing reads were first-quality filtered and then subsequently analyzed, and sequenced fragments smaller than 200bp were removed. Host DNA reads were also removed by aligning to the human reference genome (GRCh38). 4) The remaining filtered reads were subsequently compared with the TB drug resistance gene database to generate drug resistance gene analysis results. TNS detects multiple anti-tuberculosis drugs resistance genes simultaneously. The drug resistance target genes of *M.tb.* tested by TNS are shown in Table 1.

Statistical analysis

In this study, statistical analysis was performed using R (version 4.1.3). Using the clinical composite reference standard as the reference standard, the compliance rate was calculated = (number of true positive cases + number of true negative cases)/total number

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TABLE 1 List of target genes sequenced by targeted nanopore, detected target gene mutation sites and mutation frequencies.

Drug	Target gene	Mutations	No. of isolates
Rifampicin	***rpoB***	**Leu511Pro**	1
		Ser531Leu	3
		His526Tyr, Ser531Leu	1
		Thr400Ala, Ser531Leu	1
		Gln513Pro	1
		His526Tyr	1
Isoniazid	***katG***	**Ser315Thr**	5
		Thr394Ala, Ser315Thr	1
	inhA	**– 15 C→T**	1
	ahpC		0
Pyrazinamide	***pncA***	**Asp63Gly**	1
		Thr76Ile	1
		His51Arg	1
Ethambutol	***embB***	**Tyr319Ser**	1
		Asp328Tyr	1
Fluoroquinolones	***gyrB***	**Asp94Gly**	1
		Ala90Val	1
		Ser91Pro	1
Streptomycin	***rpsl***	**Lys43Arg**	4
	rrs		0
Prothionamide	***inhA***		0
Amikacin	***rrs***		0
Capreomycin	***rrs***		0
Aminosalicylic acid	***folC***		0
	thyA		0
	ribD		0

of tests × 100%; sensitivity = number of true positive cases/(number of true positive cases + number of false negative cases) × 100%; specificity = number of true negative cases/(number of true negative cases + number of false positive cases) × 100%. We evaluated the concordance between diagnostic assays with McNemar's test. A p-value <0.05 was considered statistically significant for all the analyses.

Results

Clinical characteristics

A total of 60 patients were enrolled between January and October 2022, excluding 2 patients who refused to perform bronchoscopy. The final enrollment of 58 patients was performed by TNS. Figure 1 shows the classification of patients included in this study. Patients provide one BALF specimen each, there is no HIV in

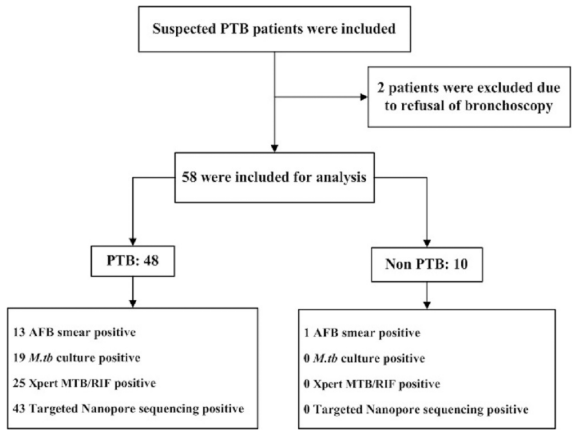

FIGURE 1
The classification flowchart of patients included in this study.

the patient. Clinical characteristics and demographics are shown in Table 2.

Consistency between targeted nanopore sequencing test and other confirmatory diagnosis methods

In this study, 30 patients with a final diagnosis of definite PTB (definite PTB was confirmed by either a positive Xpert MTB/RIF and/or *M.tb.* culture). TNS had a sensitivity of 100% (30/30) for definite PTB. *M.tb.* culture and Xpert MTB/RIF negative PTB cases were reported positive by TNS in 13 cases and were consistent with clinical diagnosis, Table 3 showed the consistency between TNS test and other confirmatory diagnosis methods among patients with PTB. Figure 2 shows the distribution and overlap of AFB smear, *M.tb.* culture, Xpert MTB/RIF, and TNS.

Diagnostic accuracy of the four tests

Using the clinical composite diagnosis as the reference gold standard, the sensitivity of AFB smear were 27.1% (95% CI: 15.3-41.8); for *M.tb* culture were 39.6% (95% CI: 25.8-54.7); for Xpert MTB/RIF were 56.2% (95% CI: 41.2-70.5); for TNS were 89.6% (95% CI: 77.3-96.5). Table 4 shows the sensitivity, specificity, PPV, and NPV data for AFB smear, *M.tb.* culture, Xpert MTB/RIF, and TNS of BALF specimens, respectively. In these four testing methods, AFB smears showed the lowest diagnostic accuracy. TNS had the highest diagnostic accuracy and was obviously better than *M.tb.* culture and Xpert MTB/RIF ($P<0.05$). Xpert MTB/RIF and *M.tb.* culture each had moderate diagnostic accuracy, there is no statistical difference between them ($P>0.05$).

Mutation sites and frequencies of relevant drug resistance genes detected by targeted nanopore sequencing

Among the patients who tested positive for TNS, 10 patients had different targeting gene mutations in *M.tb.*, Five of them tested positive for *M.tb.* culture and five tested negative for *M.tb.* culture. The relevant drug resistance gene mutation sites and frequencies detected by TNS are shown in Table 1. The *rpoB* gene detected amino acid codon mutations at 511, 513, etc. The *katG* gene detected the most codon mutations at position 315. The *pncA* gene detected codon mutations at positions 63 and 76. The *embB* detected codon mutations at positions 319 and 328. The *gyrB* gene detected codon mutations at positions 90, 91, and 94, which is highly consistent with fluoroquinolone resistance. The *rpsL* gene detected codon mutations at position 43.

Targeted nanopore sequencing compared with phenotypic DST

All 19 patients with TB positive cultures were subsequently tested for phenotypic drug susceptibility. We compared the

TABLE 2 Clinical characteristics of the included patients.

Clinical characteristics	All (n = 58)	PTB (n = 48)	Non-PTB (n = 10)
Age(year, mean ± SD)	44.60 ± 19.03	42.48 ± 19.77	54.80 ± 10.54
Gender			
Male(n, %)	40 (68.97)	33 (68.75)	7(70.00)
Female(n, %)	18(31.03)	15 (31.25)	3(30.00)
TST			
Positive	51 (87.93)	46 (95.83)	5(50.00)
ELISPOT			
Positive	49 (84.48)	44 (91.67)	5(50.00)
HIV			
Positive	0 (0.00)	0 (0.00)	0(0.00)
Complaint symptoms			
asymptomatic	9 (15.52)	8 (16.67)	1(10.00)
hemoptysis	8 (13.79)	7 (14.58)	1(10.00)
cough	22 (37.93)	19 (39.58)	3(30.00)
fever	16 (27.58)	12 (25.00)	4(40.00)
chest tightness	3 (6.17)	2 (4.17)	1(10.00)

TST, tuberculin skin test; ELISPOT, enzyme-linked immunospot; HIV, Human immunodeficiency virus.

TABLE 3 Consistency between targeted nanopore sequencing test and other confirmatory diagnosis methods.

confirmatory diagnosis methods		targeted nanopore sequencing		P value
		Positive (N=43)	Negative (N=5)	
GeneXpert MTB/RIF	**Positive**	**27 (100.0%)**	**0 (0.0%)**	**<.001**
	Negative	**16 (76.2%)**	**5 (23.8%)**	
***Mycobacterium tuberculosis* Culture**	**Positive**	**19 (100.0%)**	**0 (0.0%)**	**<.001**
	Negative	**24 (82.8%)**	**5 (17.2%)**	
GeneXpert MTB/RIF and/or *Mycobacterium tuberculosis* Culture	**Positive**	**30 (100.0%)**	**0 (0.0%)**	**<.001**
	Negative	**13 (72.2%)**	**5 (27.8%)**	

genotypic and phenotypic drug susceptibility results of the 11 antitubercular drugs.The consistency analysis of the two DST results is shown in Table 5. Phenotypic and genotypic DST of rifampicin, isoniazid, streptomycin, and fluoroquinolones had high consistency.1 case of phenotypic resistance to ethambutol, no mutation detected in TNS. 1 case of phenotypic drug susceptibility to pyrazinamide and ethambutol, *pncA* and *embB* mutation in coding gene detected by TNS.

Discussion

In this study, we evaluated the application of TNS in the diagnosis of pulmonary TB. BALF specimens were selected and compared with Xpert MTB/RIF, AFB smear, and *M.tb.* culture, and it was found that the positive detection rate of TNS was significantly higher than that of the other three methods, and the differences were statistically significant. TNS also has high sensitivity in patients with negative results from Xpert MTB/RIF tests and from TB culture tests. Therefore, for patients with negative sputum smear or low sputum volume, the detection rate of TB patients can be significantly improved by BALF examination used in conjunction with TNS. This is very significant for controlling the TB epidemic.

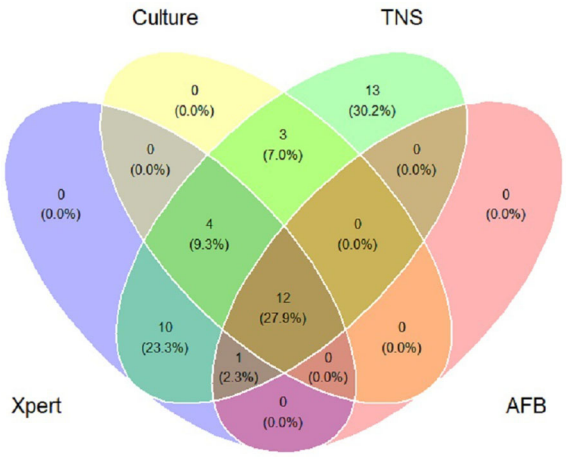

FIGURE 2
Venn diagram of positive tests for patients of pulmonary tuberculosis. Xpert: Xpert MTB/RIF; Culture: *Mycobacterium tuberculosis* culture; TNS: Targeted nanopore sequencing; AFB: acid-fast bacilli smear.

In this study, more than 10 target genes were selected for TNS, covering most of the target genes for antituberculosis drug action. In contrast to Xpert MTB/RIF, which can only detect an 81bp region of the *rpob* gene, TNS can detect drug resistance genes of longer length, such as covering the full length of the *rpob* gene. Xpert MTB/RIF was unable to distinguish between heterogeneous drug resistance and silent mutations, but TNS can distinguish them and identify them better. The genotypic and phenotypic drug susceptibility results from TNS also showed high concordance, but the genotypic drug susceptibility from TNS showed 1 month earlier than the phenotypic drug susceptibility. It is very valuable for guiding clinicians' treatment.

In this study, the mutation sites detected in the target genes of anti-TB drugs in routine use were consistent with the reported previous studies. *rpoB* gene detected amino acid codon mutations at 531 was the most common, and was highly associated with rifampicin resistance, the test results are consistent with the findings of Williams DL (Williams et al., 1998; Nakata et al., 2012). The *katG* gene detected the most codon mutations at position 315, the test results are consistent with the reported by Wengnack NL (Wengenack et al., 1997). The *rpsL* gene detected codon mutations at position 43, which is consistent with that reported by He J (He et al., 2014).

In this study, we compared the results of 19 cases of genotypic and phenotypic drug susceptibility only, which may have biased the concordance between genotypic and phenotypic drug susceptibility. This is because the number of cases with positive TB cultures and the number of drug-resistant TB cases included was low. In a follow-up study, we plan to increase the sample size and the number of TB-resistant patients for more thorough subgroup and in-depth analysis. We combined the genotypic and phenotypic drug susceptibility results in the subsequent clinical treatment, and the treatment was effective after adjusting the anti-tuberculosis regimen. In particular, phenotypic DST for pyrazinamide is not reliable (Piubello et al., 2018), so genotypic DST can compensate for the lack of phenotypic DST.

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TABLE 4 The diagnostic accuracy of the four tests for the diagnosis of pulmonary tuberculosis.

Test	Sensitivity(%)	Specificity(%)	PPV(%)	NPV(%)
AFB smear	27.1 (95% CI: 15.3-41.8)	90.9 (95% CI: 58.7-99.8)	92.9 (95% CI: 66.1-99.8)	22.2 (95% CI: 11.2-37.1)
***M.tb* culture**	39.6 (95% CI: 25.8-54.7)	100.0 (95% CI: 69.2-100.0)	100.0 (95% CI: 82.4-100.0)	25.6 (95% CI: 13.0-42.1)
Xpert MTB/RIF	56.2 (95% CI: 41.2-70.5)	100.0 (95% CI: 69.2-100.0)	100.0 (95% CI: 87.2-100.0)	32.3 (95% CI: 16.7-51.4)
Targeted nanopore sequencing	89.6 (95% CI: 77.3-96.5)	100.0 (95% CI: 69.2-100.0)	100.0 (95% CI: 91.8-100.0)	66.7 (95% CI: 38.4-88.2)

AFB, acid-fast bacilli; CI, confidence interval; M.tb, Mycobacterium tuberculosis; NPV, negative predictive value; PPV, positive predictive value.

resistance in Mycobacterium tuberculosis complex: technical guide in 2018. This guideline demonstrates that next-generation sequencing is a powerful tool to ability to improve TB management and control through rapid and accurate detection of all clinically relevant genetic mutations, leading to rapid diagnosis of drug-resistant TB bacteria in clinical specimens. This information is critical for clinicians to rapidly decide on the best therapy to treat multiple and extensively drug-resistant TB. NGS can be successfully used as a highly efficient diagnostic technique for drug-resistant TB and its performance is expected to improve as we increasingly understand the genetic basis of phenotypic resistance and the relevance of genetic resistance to the clinic.

Next-generation sequencing (NGS) has great potential as a rapid diagnostic method for drug-resistant TB in different clinical laboratory settings worldwide. While current molecular assays for drug-resistant TB primarily identify limited *M.tb.* drug-resistant mutations indirectly by hybridizing probes to specific gene sequences, NGS assays can provide detailed sequence information for multiple gene regions or entire genomes. Advances in NGS technology have made possible the routine application of targeted NGS and whole-genome sequencing (WGS) in the *Mycobacterium tuberculosis* complex, and WGS can provide a nearly complete of the *Mycobacterium tuberculosis* genome, but WGS is usually performed only on culture-positive strains because relatively

TABLE 5 Comparing genotypic drug susceptibility results of targeted nanopore versus phenotypic drug susceptibility.

Drug	Target genes & mutation sites		Phenotypically susceptible (No. of isolates)	Phenotypically resistant (No. of isolates)	Concordance (%)
Rifampicin	*rpoB*	Leu511Pro	0	1	100
	rpoB	Ser531Leu	0	2	
	rpoB	Ser531Leu, Thr400Ala	0	1	
	No mutations detected		15	0	
Isoniazid	*katG*	Ser315Thr	0	3	100
	inhA	– 15 C→T	0	1	
	No mutations detected		15	0	
Fluoroquinolones	*gyrB*	Asp94Gly	0	1	100
	gyrB	Ala90Val	0	1	
	No mutations detected		17	0	
Streptomycin	*rpsl*	Lys43Arg	0	3	100
	No mutations detected		16	0	
Pyrazinamide	*pncA*	Asp63Gly	1	0	94.7
	pncA	Thr76Ile	0	1	
	No mutations detected		17	0	
Ethambutol	*embB*	Tyr319Ser	1	0	89.5
	No mutations detected		17	1	
Amikacin	No mutations detected		19	0	-
Capreomycin	No mutations detected		19	0	-
Aminosalicylic acid	No mutations detected		19	0	-
Prothionamide	No mutations detected		19	0	-

high-quality DNA is required to generate complete WGS data. Therefore, WGS is more suitable for epidemiological investigations and various scientific studies. Targeted NGS, on the other hand, can generate *Mycobacterium tuberculosis* sequence data at specific genetic loci of interest. Since *M.tb* resistance arises mainly through point mutations in specific genetic targets, targeted NGS offers great promise for the rapid diagnosis of drug-resistant TB. Targeted NGS is achieved by analysis of drug-resistant gene amplification or hybridization/capture, allowing large sequence coverage depth for sequencing large targets. The large depth of coverage provides high confidence in mutation detection and also enables the detection of heterogeneous drug resistance in samples. Targeted NGS may yield valid results even for minimal amounts and quality of DNA input, and the technique has been successfully applied to drug resistance detection in clinical TB specimens, further reducing the time to diagnosis of TB and drug-resistant TB (World Health O, 2018).

The nanopore sequencing platform, developed by Oxford Nanopore Sequencing Technologies (ONT) (Oxford, UK), identifies DNA bases by measuring the change in conductivity generated as the DNA strand passes through a biological nanopore (MinION, 2022), which has a slightly shorter sample and library preparation steps and generally lower infrastructure requirements compared to Illumina technology. Nanopore sequencing is also increasingly used in clinical applications due to its high throughput, real-time readability, and speed of data analysis. Nanopore sequencing is increasingly being used in TB, with previous studies demonstrating its excellent accuracy in predicting *M.tb* resistance. Mariner-Llicer C et al. compared the ability of the Illumina platform and MinION sequencing platform for rapid detection of *Mycobacterium tuberculosis* drug resistance genes and found that the nanopore sequencer-based targeted sequencing approach and the Illumina platform whole genome sequencing approach. The results of the nanopore sequencing-based method and the Illumina platform whole-genome sequencing method was 100% consistent in detecting drug-resistant mutations in *Mycobacterium tuberculosis*(Mariner-Llicer et al., 2021). Tafess K et al. used a second-generation sequencing Miseq and a third-generation nanopore sequencing MinIon to target 19 genes associated with drug resistance in *Mycobacterium tuberculosis*. The average sensitivity of second-generation sequencing was 82.3% and specificity was 98.0%; nanopore sequencing had 94.8% sensitivity and 98.0% specificity, showing that nanopore sequencing covered more regions of *Mycobacterium tuberculosis* drug resistance genes with faster detection and higher detection rate (Tafess et al., 2020). The study by Guocan Yu et al. evaluated the effectiveness of nanopore sequencing of respiratory specimens in early diagnosis of pulmonary tuberculosis (PTB) while comparing it with *M.tb* culture and Xpert MTB/RIF. The sensitivity and specificity of AFB smear were 27.6% and 87.5%, respectively; the sensitivity and specificity of *M.tb.* culture were 57.8% and 100.0%, respectively; the sensitivity and specificity of Xpert MTB/RIF were 62.9% and 97.9%, respectively; and the sensitivity and specificity of nanopore sequencing were 94.8% and 97.9%, respectively, which proved that the diagnostic accuracy of nanopore sequencing for PTB The sensitivity and specificity of nanopore sequencing were 94.8% and 97.9%, respectively. Nanopore sequencing can replace Xpert MTB/RIF for the initial detection of PTB and improve the accuracy of TB diagnosis (Yu et al., 2022).

This study is exploratory and has some limitations. First, because of the limited study funding, the number of samples collected was small, which may have a biasing effect on the results, but the advantages of TNS in the etiological detection of pulmonary tuberculosis were observed from the cases included in the study. Secondly, although NGS technology can also predict drug resistance of Mycobacterium tuberculosis, the number of drug-resistant patients in the study selection is not sufficient for comparative analysis, and future studies should increase the sample size and the number of drug-resistant TB samples for more detailed grouping and in-depth analysis.

China revised the diagnostic criteria for tuberculosis in 2017, using positive results from molecular biology techniques as diagnostic criteria for pathogenically positive tuberculosis, greatly complementing traditional bacteriological detection methods (National Health, 2017). This study evaluates the potential value of TNS for the diagnosis of tuberculosis, and the application of BALF specimens for TNS has good accuracy for the diagnosis of tuberculosis, significantly better than Xpert MTB/RIF, AFB smear, and *M.tb.* culture. And the results were highly consistent with the phenotypic DST and Xpert MTB/RIF, and the results of multi-drug resistance detection could be obtained within 48 hours. With the further development and improvement of NGS technology, it gradually achieves low cost and high efficiency and is expected to become a new viable method for clinical research and diagnosis of TB and drug-resistant TB.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

Author contributions

XS, XL, FL, JS and XR designed and supervised the study. XS, QW, ZW enrolled patients and assisted in the collection of samples. XS, HW, JH, WZ performed experiments and analyzed data. XS, XR wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

Health Commission of Henan Province (Grant Number LHGJ20200214).

Conflict of interest

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

Publisher's note

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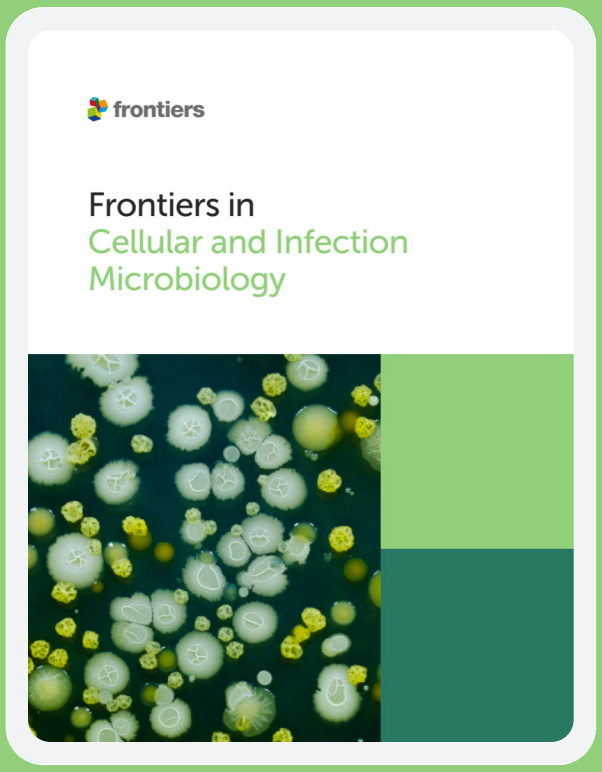

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