Journal of Infection 90 (2025) 106395

Contents lists available at ScienceDirect

Journal of Infection

journal homepage: www.elsevier.com/locate/jinf

Infectious Disease Practice

Diagnostic performance of metagenomic next-generation sequencing among hematological malignancy patients with bloodstream infections after antimicrobial therapy

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ARTICLE INFO

Article history:
Accepted 22 December 2024
Available online 27 December 2024

Keywords:
Metagenomics
Hematological malignancies
Bloodstream infections
Antimicrobial therapy
Microbial sensitivity tests

SUMMARY

Background: Metagenomic next-generation sequencing (mNGS) is an effective method for detecting pathogenic pathogens of bloodstream infection (BSI). However, there is no consensus on whether the use of antibiotics affects the diagnostic performance of mNGS. We conducted a prospective clinical study aiming to evaluate the effect of antimicrobial treatment on mNGS.

Methods: Blood samples were collected for mNGS testing within 24 h of culture-confirmed with BSI, with re-examination conducted every 2–3 days.

Results: A total of 38 patients with BSI were enrolled. The mNGS positive (mNGS-pos) rate declined sharply after the use of antibiotics, with only 17 (44.78%) patients remaining mNGS-pos while the rest were mNGS negative (mNGS-neg). The median duration of pathogen identification was significantly longer for mNGS compared to blood culture (BC) (4 days vs 1 days; P < 0.0001). A positivity duration of ≥ 3 days was an independent risk factor of septic shock (OR, 20.671; 95% CI, 1.958–218.190; P = 0.012). Patients with mNGS-pos and mNGS-neg differed by the median duration of fever (6 days vs 3 days; P = 0.038), rates of drug resistance (35.3% vs 4.8%; P = 0.017), rates of septic shock (47.1% vs 14.3%; P = 0.029), and 28-day mortality (29.4% vs 4.8%; P = 0.041).

Conclusions: The antimicrobial treatment will greatly reduce the positive rate of mNGS. The duration of mNGS is significantly longer than that of BC. The prolonged duration of mNGS suggests an increased risk of septic shock and could be identified as a high-risk factor of adverse infection outcome, requiring more aggressive anti-infective treatment measures.

Introduction

Most patients with hematological malignancies experience immunosuppression, making infections a common problem for hematologists.[1] Bloodstream infection (BSI), one of the most severe infections, poses a serious threat to patient prognosis. Blood culture (BC) is the gold standard for diagnosing BSI. However, in patients with severe sepsis, BC sensitivity remains less than 40%.[2] Traditional BC relies on viable organisms in the blood; however, the amount of viable organisms decreases rapidly after antibiotic treatment, which may affect the sensitivity of BC. Missed or misdiagnosis of pathogens may lead to inappropriate antimicrobial therapy and poor outcomes. Metagenomic next-generation sequencing (mNGS), which analyses circulating cell-free deoxyribonucleic acid (cfDNA) from blood samples, effectively detects most known pathogens.[3] Since mNGS does not require pathogens to be viable, the detection may be less affected by antibiotic treatment.[4,5] However, no consensus exists on whether antibiotic use affects the diagnostic performance of mNGS, and the optimal time window for mNGS testing remains controversial. This prospective clinical study aimed to evaluate the impact of antibiotic use on mNGS and identify the optimal timing for mNGS testing; it involved hospitalized patients with hematological malignancies and culture-confirmed BSI.

Materials and methods

Study population

Patient data were derived from a prospective and observational study of hospitalized patients with hematological malignancies

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https://doi.org/10.1016/j.jinf.2024.106395

(Chinese Clinical Trials Registry, ChiCTR2100042992). All patients were in a post-chemotherapy myelosuppressed state and received treatment at the Department of Hematology, the Affiliated Drum Tower Hospital of Nanjing University Medical School, between March 11, 2021 and February 19, 2023. This study was approved by the Medical Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School (IRB No. 2020–377-02). Patients or their family members were informed of the study in detail and signed informed consent to participate.

Study design

All patients were analyzed by BC and mNGS simultaneously on the day of fever, which was defined as day 0. Given their hematologic malignancies and immunosuppressed status, blood samples were collected after fever onset, followed by immediate empirical antimicrobial therapy. The assays performed on the collected blood samples included BC, mNGS, and a suite of conventional tests, such as Epstein-Barr virus DNA (EBV-DNA), cytomegalovirus DNA (CMV-DNA), galactomannan (G), β-D-glucan (GM), C-reactive protein, and procalcitonin assays. Once the patient was culture-confirmed with BSI, an additional blood sample for mNGS testing was collected within 24 h, with repeated mNGS testing every day for the next 3 days. Afterwards, mNGS re-examination was conducted every 2–3 days until negative results were obtained. The duration of positivity for BC or mNGS was counted from day 0 (Fig. 1).

Clinical data

Physicians collected clinical data for each patient through case report forms. The information collected includes demographic characteristics, past comorbidities, and results of clinical laboratory tests. Time to positivity for BC refers to the time interval from sample collection to the occurrence of a positive culture result. Sepsis shock was defined as sepsis accompanied by hypotension (systolic blood pressure ≤ 90 mmHg) and abnormal hemodynamic perfusion.[6] Eastern Cooperative Oncology Group Performance Status (ECOG PS) scores were calculated on the day of the index positive BC.

Clinical sample collection and DNA extraction

BCs were tested by the Department of Laboratory Medicine at Nanjing Drum Tower Hospital using standard techniques for species identification of bacterial isolates. DNA extraction and library preparation were performed using an NGS Automatic Library Preparation System (Matridx Biotechnology Co., Ltd.; Hangzhou, China). The quality of DNA was assessed using a BioAnalyzer 2100 (Agilent Technologies; Santa Clara, CA, United States) combined with quantitative PCR to measure the adapters before sequencing.

Metagenomic next-generation sequencing

Qualified DNA libraries were pooled and sequenced on the Illumina NextSeq500 system (50 bp single-end; San Diego, CA, United States). Negative and positive controls were conducted in parallel to control the quality of each sequencing run. A total of 10–20 million reads were generated for each sample. The raw sequenced reads underwent quality control to remove short (length < 35 bp), low quality, and low complexity reads and those corresponding to adapters. Host sequences were filtered out based on the alignment to the human-specific database in NCBI using Bowtie2 (version 2.3.5.1). The clean reads were aligned to a manually curated microbial database using Kraken2 (version 2.1.2; confidence = 0.5) for rapid taxonomic classification. The classified reads of interested microorganisms were further validated through a second alignment to the microbial database using Bowtie2. The candidate reads were classified using BLAST (version 2.9.0) whenever the results of Kraken2 and Bowtie2 were inconsistent. mNGS analysis for each sample was completed within 24 h of sample collection.[7–10]

Pathogen identification

A panel of clinical experts, including three experienced physicians and a clinical microbiologist, evaluated the etiological screening results of patients. mNGS results were interpreted according to the standard data processing workflow of MatriDx Biotechnology Co., Ltd. Infectious agents were identified based on microbiological tests, mNGS results and clinical review results. mNGS reporting criteria required the negative control (NC) in the same sequencing run to exclude the species or the RPM (sample)/RPM (NC) ≥ 5, which was determined according to previous studies as a cutoff for discriminating true-positives from background contaminations.[11]

Statistical analyses

Continuous variables are represented as medians and quartiles, while categorical variables are presented as counts and percentages. Comparisons of continuous variables between groups were conducted using the Mann–Whitney test, while categorical variables were analyzed with the chi-squared test. Independent risk factors for septic shock were identified using binary logical regression analysis, with odds ratios and 95% confidence intervals (CI). Survival curves for fever and positivity duration were compared using the Breslow (Wilcoxon) test. Statistical analyses were conducted using SPSS software (version 22.0) and GraphPad Prism 5.0 (GraphPad Software). Statistical significance was set at $P < 0.05$.

Results

Clinical characteristics

This clinical study enrolled 38 patients with positive BCs, including 21 with acute myeloid leukemia, 1 with myelodysplastic syndrome, 8 with acute lymphoblastic leukemia, 5 with non-Hodgkin lymphoma, and 3 with multiple myeloma (Table 1). All patients were in a myelosuppressed state, and their median neutrophil count and lymphocyte count were 0 (0, 0.15) $\times 10^9$/L and 0.1 (0, 0.4) $\times 10^9$/L, respectively (Table 1). The median time from BC sample collection to the reporting of positive BC result was 11.9 (9.5, 13.1) h, approximately equal to the time of antibiotic use before the second mNGS test was submitted.

Pathogens characteristics

Among 38 patients, 39 positive pathogens result were identified by BC, including one patient with two kinds of bacteria polymicrobial infection on day 0 (before antibiotic use). All 39 pathogens identified by BC were found in mNGS on day 0. In addition to results consistent with BC, mNGS detected an additional *Pneumocystis jirovecii* in 1 patient, *Aspergillus fumigatus* in 2 patients, *Aspergillus flavus* in 1 patient, *Klebsiella pneumoniae* in 1 patient, and additional viruses in 14 patients. These viruses included cytomegalovirus (7 cases), Epstein-Barr virus (4 cases), JC polyomavirus (3 cases), BK polyomavirus (1 case), human herpesvirus type 1 (HHV-1) (3 cases), HHV-2 (1 case), HHV-6 (1 case), HHV-7 (1 case), and TS polyomavirus (1 case) (Table 2). However, the positive rate of mNGS dropped sharply after antibiotic use. Only 17 patients remain mNGS positive (mNGS-pos), while the others were mNGS negative (mNGS-neg). The positive rate of mNGS after antimicrobial therapy was only 44.7% (17/38). Baseline characteristics of the mNGS-pos or mNGS-neg group showed no significant differences (Table 1). The

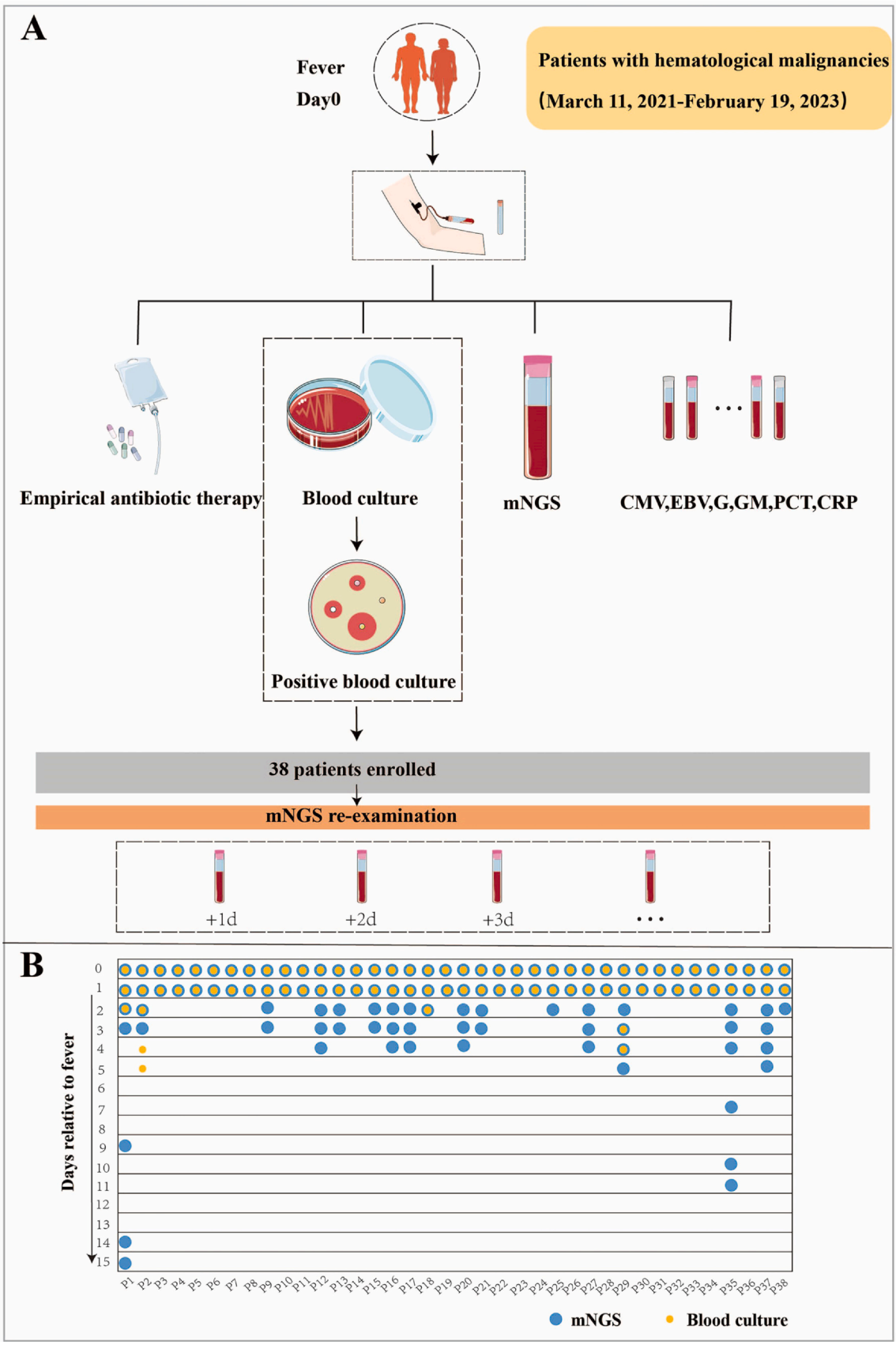

Fig. 1. (A) Study workflow. (B) The timeline of sample distribution. P indicates patient. For example, Day 0 indicates the day of fever. Day 3 indicates the third day after the onset of fever. Abbreviations: mNGS, metagenomic next-generation sequencing; EBV, Epstein-Barr virus; CMV, cytomegalovirus; G, galactomannan; GM, β-D-glucan; PCT, procalcitonin; CRP, C-reactive protein. Parts of this figure were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

Table 1
Clinical characteristics of the study population.

	Overall Study Cohort n=38		
	mNGS-pos n = 17	mNGS-neg n = 21	P
Age (years), median (Q1, Q3)	52 (28, 61)	51 (29.5, 56.5)	0.777
Sex (male), n (%)	11 (64.7)	11 (52.4)	0.523
Diagnosis, n (%)			
AML	9 (52.9)	12 (57.1)	0.798
MDS	0	1 (4.8)	0.386
ALL	4 (23.5)	4 (19.0)	0.740
NHL[a]	2 (11.8)	3 (14.3)	0.822
MM	2 (11.8)	1 (4.8)	0.432
Comorbidities			
Diabetes mellitus, n (%)	3 (17.6)	3 (14.3)	0.780
Autoimmune disease, n (%)	1 (5.9)	1 (4.8)	0.879
ECOG PS, n (%)			
0	4 (23.5)	6 (28.6)	0.729
1	10 (58.8)	12 (57.1)	0.918
2	2 (11.8)	3 (14.3)	0.822
3	1 (5.9)	0	0.266
Corticosteroid use (within 14 days), n (%)	7 (41.2)	7 (33.3)	0.623
Allo-HSCT recipient, n (%)	5 (29.4)	5 (23.8)	0.772
Neutrophil count ($\times10^9$/L), median (Q1, Q3)	0 (0, 0.1)	0 (0, 0.6)	0.351
Lymphocyte count ($\times10^9$/L), median (Q1, Q3)	0.1 (0, 0.4)	0.2 (0.1, 0.5)	0.311
Prophylactic Treatment, n (%)	11 (64.7)	17 (81.0)	0.264
Etiology characteristics			
Time to positivity for blood culture[b] (hours), median (Q1, Q3)	11.6 (6.7, 12.7)	12.1 (10.2, 13.2)	0.223
Drug resistance, n (%)	6 (35.3)	1 (4.8)	**0.017**
Failed empirical therapy, n (%)	7 (41.2)	5(23.8)	0.258
Outcomes of infection			
Septic shock, n (%)	8 (47.1)	3 (14.3)	**0.029**
28-day mortality, n (%)	5 (29.4)	1 (4.8)	**0.041**
Antipyretic time (days), median (Q1, Q3)	6 (4.0, 11.0)	3 (2.0, 9.5)	**0.038**
Duration of antibiotic administration (days), median (Q1, Q3)	11(8.0, 35.5)	14 (8.0, 17.0)	0.445

Abbreviations: mNGS-pos, metagenomic next-generation sequencing positive; mNGS-neg, metagenomic next-generation sequencing negative; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; ECOG PS, Eastern Cooperative Oncology Group Performance Status; Allo-HSCT, allogeneic hematopoietic stem cell transplantation.
Bold value indicates significant value (P-value < 0.05).
[a] NHL included mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL) and Burkitt lymphoma (BL).
[b] Time interval from BC sample collection to the occurrence of a positive culture result.

characteristics of the pathogenic microorganisms of BSI are listed in Table 3 and Fig. 2. Overall, 12 bacteria and 1 fungus were identified by BC, with *Escherichia coli* and *Klebsiella pneumoniae* being the most frequent bacterial pathogens. Additionally, 7 (17.9%) drug-resistance cases were identified among the 39 pathogenic microorganisms, including 3 (7.7%) carbapenem-resistant *Klebsiella pneumoniae* (CRKP), 2 (5.1%) pan-drug resistant *Acinetobacter baumannii* (PDRAB), 1 (2.6%) methicillin-resistant *Staphylococcus aureus* (MRSA) and 1 (2.6%) azole-resistant Candida. The rate of drug resistance was significantly higher in the mNGS-pos group than in the mNGS-neg group (35.3% vs 4.8%; P = 0.017; Table 1).

Antibiotic utilization protocols

Consistent with the Infectious Diseases Society of America (IDSA) guidelines,[12] 28 of the 38 patients received antibiotic prophylaxis before developing a fever, with 11 patients (64.7%) in the mNGS-pos group and 17 (81.0%) in the mNGS-neg group, demonstrating no significant difference between the two groups (Table 1, Table 2). The prophylactic regimens targeted a spectrum of pathogens, including bacteria, fungi, viruses, and *Pneumocystis jirovecii*. Following the onset of fever, the initial empirical antibiotic therapy was predominantly targeted against Gram-negative bacteria (GNB), with a subset of patients also receiving concomitant therapy against Gram-positive bacteria and fungi. Based on the BC and antimicrobial susceptibility data, empirical treatment failed to cover the causative pathogens in 12 patients (7 [41.2%] in the mNGS-pos group and 5 [23.8%] in the mNGS-neg group). Although the failure rate was higher in the mNGS-pos group, the difference was not statistically significant (Table 1, Table 2). The duration of antibiotic administration did not differ significantly between these two groups (Table 1). We hypothesized that it might be related to the higher mortality rate in the mNGS-pos group, which experienced rapid disease progression to death; thus, the duration of antibiotic administration may not truly reflect the disease severity.

Duration of positivity

Of the 17 patients who were mNGS-pos, 3 remained positive for 2 days after the use of antibiotics, 5 for 3 days, 7 for 4 days, and 2 after 10 days (Fig. 3). The median duration of positivity was significantly longer for mNGS (4 days, IQR 3–4) compared to BC (1 day, IQR 1–1) (P < 0.0001, Breslow test; Fig. 4). The median duration of positivity for mNGS in patients with septic shock was 4 (0, 5) days, significantly longer than that in patients without shock, which was 0 (0, 2) days (P = 0.003, Breslow test; Fig. 5).

Risk factors for septic shock

Independent risk factors for septic shock were investigated through multivariate binary logistic regression analysis (Table 4). The duration of pathogen identification by mNGS ≥ 3 days was one of the independent risk factors of septic shock (OR, 20.671; 95% CI, 1.958–218.190; P = 0.012). Additionally, corticosteroid-containing chemotherapy regimens used within 14 days prior to fever were associated with a higher risk of septic shock (OR, 9.430; 95% CI, 1.477–60.199; P = 0.018).

Persistent positive results of mNGS and infection outcome

The median duration of fever in the mNGS-pos group was 6 (4, 11) days, significantly longer than that in the mNGS-neg group which was 3 (2, 9.5) days (P = 0.038 by Breslow test; Table 1, Fig. 6). Patients with mNGS-pos and mNGS-neg differed by rates of drug resistance (mNGS-pos 35.3% vs mNGS-neg 4.8%; P = 0.017), rates of septic shock (mNGS-pos 47.1% vs mNGS-neg 14.3%; P = 0.029), and 28-day mortality (mNGS-pos 29.4% vs mNGS-neg 4.8%; P = 0.041) (Table 1, Fig. 7).

Factors influencing the prognosis of anti-infective treatment include whether the initial empirical therapy can successfully cover the causative pathogens. Compared to the 26 patients with successful empirical treatment (emp-succ group), the 12 patients with failed empirical treatment (emp-fail group) had a longer duration of fever [9 (4.3, 14.0) days vs 4 (2.0, 7.0) days; P = 0.029], and higher 28-day mortality (33.3% in emp-fail group vs 7.7% in the emp-succ group; P = 0.047) (Table 2, Table 5).

Discussion

BSI is a serious infectious disease caused by pathogenic microorganisms entering the bloodstream and is a common complication in patients with hematological malignancies, predisposing patients to septic shock and death. BC is the generally accepted gold standard for diagnosing BSI; however, it is time-consuming and usually has a sensitivity of less than 30%. BC relies on microbiological growth, meaning that empirical antibiotic therapy may produce false-

Table 2
Etiology and antibiotics information of the study population.

Patient ID	Group	BC	mNGS			Prophylactic Treatment (Prior to Day 0)	Empirical treatment (Pre-result of BC)	Duration of antibiotic administration (d) (Post-Day 0)[a]
			Bacteria (reads)	Fungi (reads)	Virus (reads)			
Pt 01	mNGS-pos/ emp-fail	*Candida (azole-resistance)*	-	Candida	-	VOR	MEM	89
Pt 02	mNGS-pos/ emp-fail	*Klebsiella pneumoniae (CRKP)*	*Klebsiella pneumoniae*	-	CMV (1)*	LFX + Posaconazole	IPM	28
Pt 03	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	-	CAZ	BIA	11
Pt 04	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	-	LFX + FCA	BIA	12
Pt 05	mNGS-neg/ emp-fail	*Stretpococcus mitis*	*Stretpococcus mitis*	-	-	VOR	BIA	10
Pt 06	mNGS-neg/ emp-fail	*Staphylococcus epidermidis*	*Staphylococcus epidermidis*	*PJ* (10)*	EBV (1)* CMV (1)*	LFX + VOR	TZP	26
Pt 07	mNGS-neg/ emp-succ	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	-	CMV (4)*	LFX + FCA	IPM	7
Pt 08	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	-	MXF + VOR	MEM + CAS	20
Pt 09	mNGS-pos/ emp-fail	*Klebsiella pneumoniae (CRKP)*	*Klebsiella pneumoniae*	-	-	-	MEM	8 (Deceased)
Pt 10	mNGS-neg/ emp-fail	*Acinetobacter baumannii (PDRAB)*	*Acinetobacter baumannii*		-	CAZ + FCA	MEM + LNZ + CAS	16 (Deceased)
Pt 11	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	-	LFX + VOR	MEM	14
Pt 12	mNGS-pos/ emp-succ	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	-	JC polyomavirus (1)*	LFX + FCA	BIA	10
Pt 13	mNGS-pos/ emp-fail	*Staphylococcus aureus (MRSA)*	*Staphylococcus aureus*	-	CMV (3)* HHV-6 (10)*	-	IPM	5 (Deceased)
Pt 14	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	-	-	BIA	7
Pt 15	mNGS-pos/ emp-succ	*Morganella morganii*	*Morganella morganii*	*Aspergillus fumigatus* (4)*	-	VOR	MEM	36
Pt 16	mNGS-pos/ emp-succ	*Enterobacter cloacae*	*Enterobacter cloacae*	-	JC polyomavirus (2)*	-	BIA	30
Pt 17	mNGS-pos/ emp-succ	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	*Aspergillus flavus* (4)*	EBV (44)* HHV-1 (2)*	CAZ + FCA	MEM + VOR	6 (Deceased)
Pt 18	mNGS-neg/ emp-fail	*Stretpococcus mitis*	*Stretpococcus mitis, Klebsiella pneumoniae* (1)*	-	-	CEC	MEM	15
Pt 19	mNGS-pos/ emp-fail	*Acinetobacter baumannii (PDRAB)*	*Acinetobacter baumannii*		-	-	IPM	11 (Deceased)
Pt 20	mNGS-pos/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	-	-	BIA	10
Pt 21	mNGS-pos/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	EBV (83)*	CEC + VOR	BIA	41
Pt 22	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	-	-	BIA + VAN	9
Pt 23	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	HHV-1 (2)*	-	IPM	6
Pt 24	mNGS-neg/ emp-succ	*Salmonella enteritidis*	*Salmonella enteritidis*	-	EBV (1)*	-	IPM	18
Pt 25	mNGS-pos/ emp-fail	*Klebsiella pneumoniae (CRKP)*	*Klebsiella pneumoniae*	-	BK polyomavirus (2)* HHV-1 (1)*	CAZ + Posaconazole	CSL + CAS	11 (Deceased)
Pt 26	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	-	LFX + VOR	MEM	7
Pt 27	mNGS-pos/ emp-succ	*Klebsiella variicola*	*Klebsiella variicola*	-	CMV (18)*	LFX	BIA	5
Pt 28	mNGS-neg/ emp-succ	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	-	-	CEC + VOR	BIA	19
Pt 29	mNGS-pos/ emp-succ	*Bacillus cereus*	*Bacillus cereus*	-	-	-	IPM	8
Pt 30	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	CMV (7)* HHV-2 (14)* HHV-7 (7)*	LFX	IPM + VOR	16
Pt 31	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	–	MXF + VOR	BIA	16
Pt 32	mNGS-neg/ emp-fail	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Aspergillus fumigatus* (3)*	-	MXF, Micafungin	BIA	13
Pt 33	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	-	-	CAZ + VOR	BIA	14
Pt 34	mNGS-neg/ emp-succ	*Escherichia coli*	*Escherichia coli*	–		CEC + SMZ + VOR + ACV	IPM	23
Pt 35	mNGS-pos/ emp-succ	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	-	-	CEC + SMZ + VOR + ACV	MEM	35

(*continued on next page*)

Table 2 (*continued*)

Patient ID	Group	BC	mNGS			Prophylactic Treatment (Prior to Day 0)	Empirical treatment (Pre-result of BC)	Duration of antibiotic administration (d) (Post-Day 0)[a]
			Bacteria (reads)	Fungi (reads)	Virus (reads)			
Pt 36	mNGS-neg/ emp-succ	*Enterobacter cloacae*	*Enterobacter cloacae*	-	-	MXF	IPM	6
Pt 37	mNGS-pos/ emp-fail	*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*	*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*	-	TS polyomavirus (58)* JC polyomavirus (2)* CMV (1)*	Posaconazole	IPM	40
Pt 38	mNGS-pos/ emp-succ	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*	-	-	CEC + SMZ + VOR + ACV	IPM	20

Abbreviations: Pt, patient; BC: blood culture; mNGS, metagenomic next-generation sequencing; emp-succ: successful empirical treatment; emp-fail: failed empirical treatment; CMV: cytomegalovirus; EBV: Epstein-Barr virus; HHV: human herpesvirus; PJ: *Pneumocystis jirovecii*; SMZ: sulfamethoxazole; VOR: Voriconazole; MEM: Meropenem; IPM: Imipenem; LFX: Levofloxacin; CAZ: Ceftazidime; BIA: Biapenem; TZP: Piperacillin-Tazobactam; MXF: Moxifloxacin; FCA: Fluconazole; CAS: Caspofungin; LNZ: Linezolid; CEC: Cefaclor; VAN: Vancomycin; CSL: Cefoperazone-Sulbactam; ACV: Acyclovir; CRKP: carbapenem-resistant *Klebsiella pneumoniae*; PDRAB: pan-drug resistant *Acinetobacter baumannii*; MRSA: methicillin-resistant *Staphylococcus aureus*.

*Additional pathogens identified by mNGS beyond BC.

[a] A period of 12 h or more is equated to one full day.

Table 3
Microbiologic characteristics of the BSI study population.

Microbiologic Characteristic	Overall n = 39[a] (%)	mNGS-pos n = 18 (%)	mNGS-neg n = 21 (%)
Gram-negative bacteremia			
Escherichia coli	14 (35.9)	2 (5.1)	12 (30.8)
Klebsiella pneumoniae	10 (25.6)	8 (20.6)	2 (5.1)
Drug resistance		3 (7.7)[b]	-
Enterobacter cloacae	2 (5.1)	1 (2.6)	1 (2.6)
Acinetobacter baumannii	2 (5.1)	1 (2.6)	1 (2.6)
Drug resistance		1 (2.6)[c]	1 (2.6)[c]
Pseudomonas aeruginosa	2 (5.1)	1 (2.6)	1 (2.6)
Klebsiella variicola	1 (2.6)	1 (2.6)	-
Morganella morganii	1 (2.6)	1 (2.6)	-
Salmonella enteritidis	1 (2.6)	-	1 (2.6)
Gram-positive bacteremia			
Staphylococcus aureus	1 (2.6)	1 (2.6)	-
Drug resistance		1 (2.6)[d]	-
Staphylococcus epidermidis	1 (2.6)	-	1 (2.6)
Bacillus cereus	1 (2.6)	1 (2.6)	-
Stretpococcus mitis	2 (5.1)	-	2 (5.1)
Fungemia			
Candida	1 (2.6)	1 (2.6)	-
Drug resistance		1 (2.6)[e]	-

Abbreviations: BSI, Bloodstream infection; mNGS-pos, metagenomic next-generation sequencing positive; mNGS-neg, metagenomic next-generation sequencing negative.

[a] total of 38 patients, including one patient with two kinds of bacteria polymicrobial infection.

[b] Three of eight Klebsiella pneumoniae cases in mNGS-pos group were carbapenem-resistant *Klebsiella pneumoniae* (CRKP).

[c] Both the two Acinetobacter baumannii cases were pan-drug resistant *Acinetobacter baumannii* (PDRAB).

[d] The Staphylococcus aureus case was methicillin-resistant *Staphylococcus aureus* (MRSA).

[e] The *Candida* case was azole-resistance.

negative results.[2,13,14] Unlike conventional BC, mNGS analyses cfDNA from plasma samples do not hinge on culturing live organisms from blood, addressing the limitations of BC.[5,15–18] Since the impact of antimicrobial treatment on the diagnostic efficacy of mNGS and the optimal timing of mNGS in BSIs are still controversial, we performed this prospective clinical study to evaluate mNGS in patients with concurrent BSI and made four important discoveries.

Firstly, we found that the positive rate of mNGS significantly decreased after antibiotic use. Although the median time of antibiotic use before the second mNGS test was approximately 11.9 h, the positive rate of mNGS was greatly affected and dropped below 50%. While previous studies have suggested that mNGS analyses cfDNA from plasma samples do not hinge on culturing live organisms from blood,[5,15–18] our study suggests that the sensitivity of mNGS can be reduced after the antimicrobial treatment. We speculate this may be related to the short half-life of pathogenic microorganism nucleic acids in human blood. Currently, no systematic study exists on the half-life of free pathogenic microbial DNA or RNA in the human body. Dennis et al.[19] reported that in most cases, the concentration of circulating fetal DNA in the plasma of pregnant women is undetectable within 2 h after delivery, with a mean half-life of 16.3 min. Plasma nucleases and other organ systems were involved in fetal DNA clearance. The half-life of circulating tumor DNA (ctDNA) in the human body is less than 2.5 h.[20–22] Unlike human cfDNA, microbial cfDNA cannot be protected from degradation by binding to histones. Therefore, the half-life of microbial cfDNA may even be shorter than that of human cfDNA.[23,24] Christina Hartwig et al. found that the level of pathogen-derived cfDNA changed rapidly during acute sepsis in mice, suggesting its short half-life.[25] We believe that after antibiotics kill pathogenic microorganisms, the released nucleic acid fragments are quickly eliminated from the human body, decreasing the detection efficiency of mNGS.

Secondly, we discovered that even if the detection efficiency of mNGS is reduced after the antimicrobial treatment, its duration of pathogen identification is significantly longer than that of

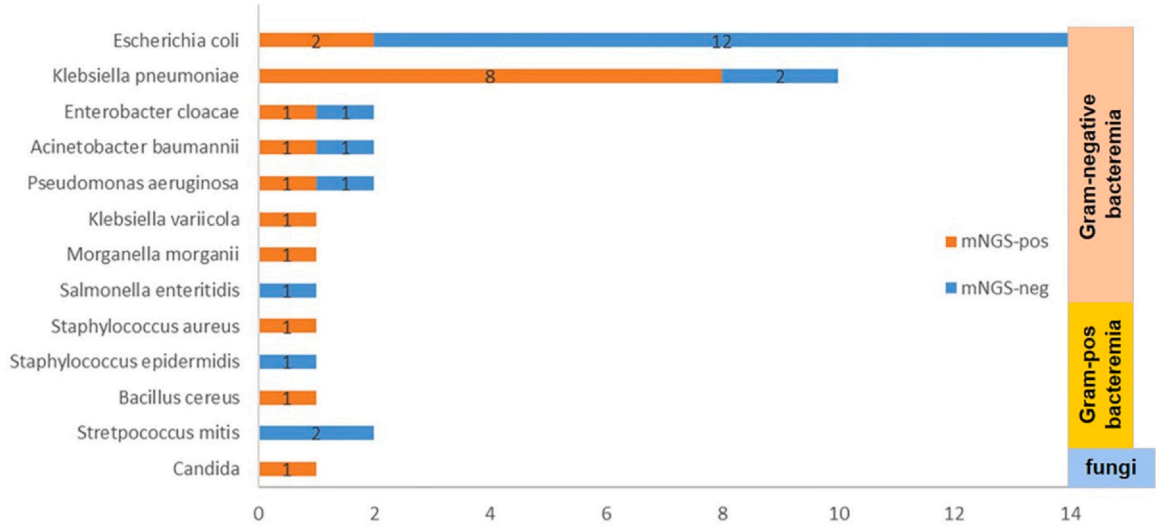

Fig. 2. Pathogen distribution in patients with culture-confirmed bloodstream infection. Abbreviations: mNGS-pos, metagenomic next-generation sequencing positive; mNGS-neg, metagenomic next-generation sequencing negative.

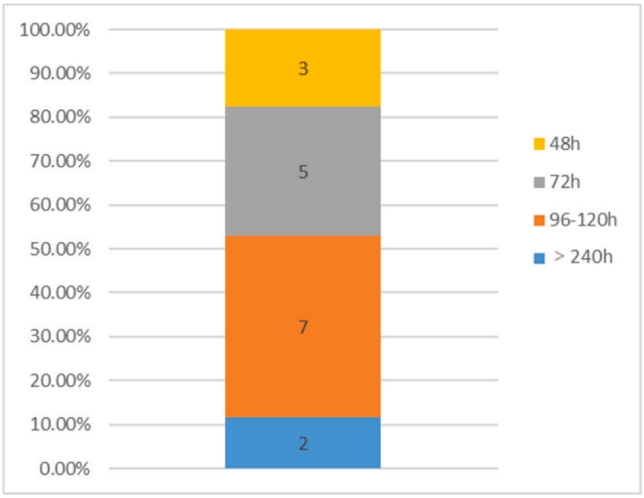

Fig. 3. Of the 17 patients who were mNGS-positive, 3 remained positive for 2 days after the use of antibiotics, 5 for 3 days, 7 for 4 days, and 2 were still positive after 10 days.

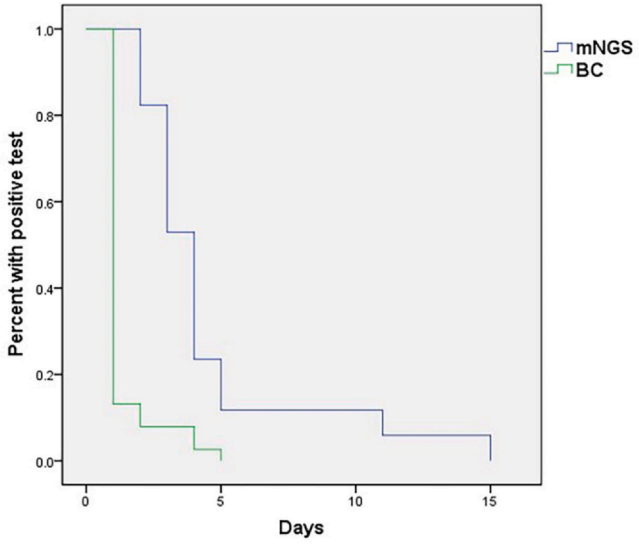

Fig. 4. Kaplan-Meier curve showing duration of positivity for mNGS (n= 17) vs BC (n=38). The median duration of positivity was significantly longer for mNGS (4 days, IQR 3–4) compared to BC (1 day, IQR 1–1) (P < 0.0001 by Breslow test). Abbreviations: BC, blood culture; mNGS, metagenomic next-generation sequencing.

conventional BC, with a median duration of 4 days after the antibiotic therapy was induced and lasting up to 15 days (Figs. 3 and 4). This result aligns with previous research,[26,27] suggesting that even after broad-spectrum antibiotic therapy, mNGS still has an advantage over BC, which depends entirely on viable pathogens. Therefore, mNGS could still be recommended after antimicrobial treatment to diagnose accurately in culture-negative patients.

Thirdly, we found that the duration of pathogen identification by mNGS in patients with BSI was associated with the occurrence of septic shock. Our finding suggested that the duration of mNGS was longer in patients with septic shock than in patients without shock, and a duration of at least 3 days was one of the independent risk factors of septic shock (Fig. 5, Table 4). Eichenberger et al. found that the duration of pathogen identification by mNGS was associated with an increased risk of metastatic infection.[27] Therefore, we believe that persistent mNGS positivity reflects the presence of pathogens with continuous proliferative activity within the body, which is related to the severity of BSIs and the effectiveness of antimicrobial therapy. Our finding indicates that mNGS duration can assist clinicians in predicting infection outcomes and promptly adjusting antibiotic therapy.

Finally, we found that persistent mNGS positive results after antibiotic therapy not only predicted septic shock but also identified a high risk of adverse infection outcomes. Our study demonstrated that compared with the mNGS-neg group, the mNGS-pos group had a longer duration of fever, higher rate of septic shock, and 28-day mortality (Table 1, Fig. 5, Fig. 6). Moreover, the drug resistance rate was higher in the mNGS-pos group than in the mNGS-neg group. Our study revealed a higher prevalence of pathogen non-coverage by empirical antimicrobial regimens in the mNGS-pos group compared to the mNGS-neg group. While this difference did not achieve statistical significance, we believed this finding was noteworthy. Table 5 shows that the emp-fail group exhibited a higher incidence of drug resistance than the emp-succ group, likely associated with adverse outcomes, such as prolonged fever duration and increased mortality rates. These findings align with previous reports.[28,29] Persistent

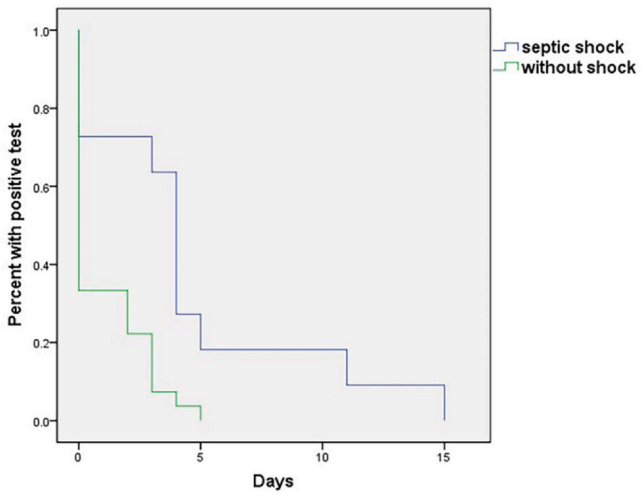

Fig. 5. Kaplan-Meier curve illustrating the duration of positivity for mNGS in patients with septic shock (n=11) and patients without shock (n=27). Median duration of mNGS was longer in patients with septic shock than in patients without shock (4 days vs 0 day; P = 0.003 by Breslow test). Abbreviations: mNGS, metagenomic next-generation sequencing.

Table 4
Multivariate binary logistic regression analysis of risk factors for septic shock.

Risk Factor	OR	95% CI	P
Age (≥60 y)	0.408	0.032 - 5.229	0.491
Acute Leukemia	10.952	0.551 - 217.872	0.117
Duration of mNGS positivity (≥3 days)	20.671	1.958 - 218.190	**0.012**
Corticosteroid use (within 14 days)	9.430	1.477 - 60.199	**0.018**

Abbreviations: mNGS, metagenomic next-generation sequencing.
Bold value indicates significant value (P-value < 0.05).

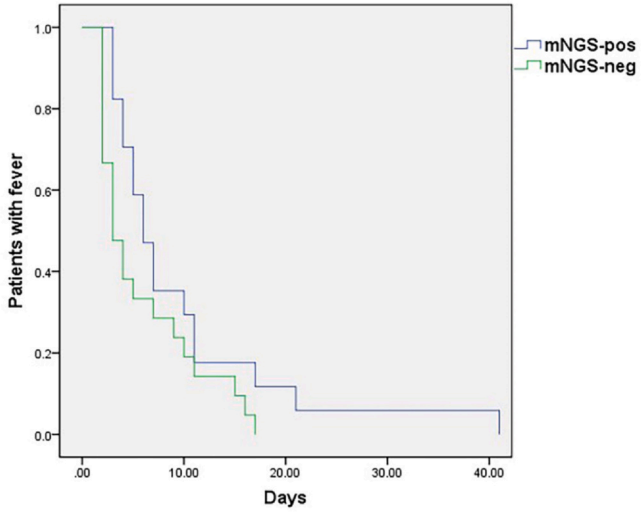

Fig. 6. Kaplan-Meier curve showing the duration of fever for mNGS-pos group (n=17) vs mNGS-neg group (n=21). The median duration of fever in the mNGS-pos group was 6 (4, 11) days, significantly longer than that in the mNGS-neg group which was 3 (2, 9.5) days (P = 0.038 by Breslow test). Abbreviations: mNGS-pos, metagenomic next-generation sequencing positive; mNGS-neg, metagenomic next-generation sequencing negative.

mNGS-positive results after antibiotic therapy may indicate antimicrobial resistance and failure of initial empirical antibiotic treatment. For example, pathogens like carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and pan-drug-resistant *Acinetobacter baumannii*

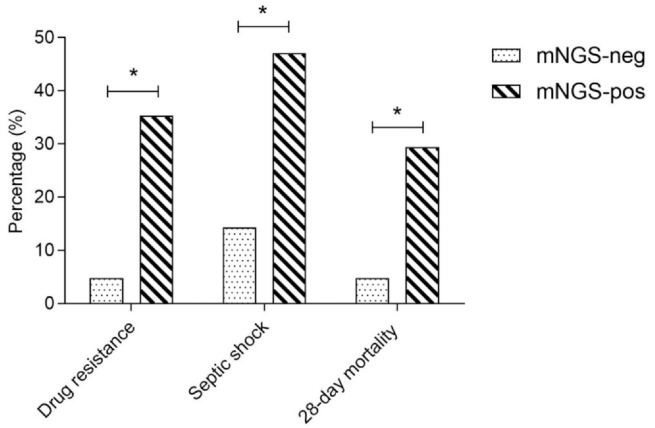

Fig. 7. The rates of drug resistance, rates of septic shock, and 28-day mortality of mNGS-pos group (n=17) vs mNGS-neg group (n=21). Abbreviations: mNGS-pos, metagenomic next-generation sequencing positive; mNGS-neg, metagenomic next-generation sequencing negative. * P < 0.05.

Table 5
Correlation between empirical therapy and patient prognosis.

	emp-succ group n = 26	emp-fail group n = 12	P
mNGS-pos, n (%)	10 (38.5)	7 (58.3)	0.258
Drug resistance, n (%)	0	7 (58.3)	**0.000**
Septic shock, n (%)	8 (30.8)	3 (25.0)	0.719
28-day mortality, n (%)	2 (7.7)	4 (33.3)	**0.047**
Antipyretic time (days), median (Q1, Q3)	4 (2.0, 7.0)	9 (4.3, 14.0)	**0.029**

Abbreviations: mNGS-pos, metagenomic next-generation sequencing positive; emp-succ: successful empirical treatment; emp-fail: failed empirical treatment.
Bold value indicates significant value (P-value < 0.05).

(PDRAB) constitute major public health threats and are strongly associated with high mortality rates. Therefore, early identification of these pathogens and their resistance pattern is essential for timely adjustments to antibiotics selection.[30–32] mNGS outperformed conventional BC in identifying these pathogens, suggesting that patients with persistent levels of detectable cfDNA might benefit from the early optimization of antibiotic therapy.

In our study, mNGS identified additional pathogens beyond those detected by BC, comprising 1 case of *Pneumocystis jirovecii*, 3 fungal infections, 1 bacterial infection, and 14 viral cases (Table 2). Clinicians determined that the bacteria and viruses were not causative pathogens. Nevertheless, we considered *Pneumocystis jirovecii*, *Aspergillus fumigatus*, and *Aspergillus flavus* as clinically significant despite their low sequence read abundance, and adjusted the antimicrobial therapy in accordance with the mNGS findings. Our study demonstrated that mNGS possesses a substantial advantage over BC in detecting rare pathogens, such as fungi and *Pneumocystis jirovecii*, consistent with previous reports.[33]

This study has some limitations that warrant further exploration. First, the sample size was small, which reduced the statistical analysis capacity. Additionally, the decrease in mNGS positivity rates following antibiotic treatment was significantly higher than in previous studies.[26,27] We hypothesize several contributing factors. First, the type of pathogen plays a role. Our study involved patients with hematological disorders experiencing bone marrow suppression, with over 80% of infections attributed to GNB. Contrastingly, Grumaz et al.[26] reported that 48% (33/67) of their cohort exhibited Gram-positive bacteremia. Similarly, Eichenberger et al.[27] documented that nearly half of their participants (66/140) had *Staphylococcus aureus* bacteremia (SAB). Their data revealed that the persistence of cfDNA

positivity in the SAB group was markedly extended compared to the GNB group (P < 0.0001). They surmised that this discrepancy may be attributed to the distinct pathophysiological characteristics and therapeutic responses associated with these two categories of infections.[27] Secondly, it may be related to the different response rates of pathogens to empirical treatments. In our study, the patient characteristics were relatively consistent, comprising individuals with malignant hematological disorders during the post-chemotherapy myelosuppressive phase. Consequently, the administration of empirical antibiotic therapy could be guided by the IDSA clinical practice guidelines.[12] Within the scope of our investigation, the empirical treatment achieved a coverage rate of 68.4% (26/38). The emp-fail group exhibited a propensity for persistent positivity in mNGS, contrasting with the emp-succ group (Table 5). However, Grumaz and Eichenberger did not address the selection of empirical antibiotic protocols, bacterial resistance prevalence, or the coverage rate of empirical treatments for pathogens. Grumaz[26] noted that 40% of the patients underwent antibiotic escalation based on mNGS results. We speculate that the high coverage rate of empirical treatment may be one of the reasons for the rapid decrease in mNGS positivity after antibiotic treatment in our study. Thirdly, the observed differences may be attributed to variations in the mNGS assay protocols. For instance, 57.1% (12/21) of the mNGS-neg group in our study were identified as *Escherichia coli*. As mentioned in the "Materials and Methods" section, we established a threshold for identifying microorganisms in samples as those with an RPM five times higher than that in the NC. For *E. coli* assessment, we incorporated an additional bioinformatics quality control method using a background sequence alignment that integrates the genomes of commonly used *E. coli* expression strains such as BL21. If the reads for *E. coli* exceed 90%, the sequence is considered a reagent background microorganism and not reported as the causative pathogen. To mitigate potential interference in the experimental workflow, we conducted a retrospective analysis of the results for these patients. In the mNGS-neg group, 33.3% (7/21) exhibited sequences of pathogens concordant with those identified through BC; however, these sequences were not reported as causative because their quantity fall below the NC threshold. This subset included sequences from five cases of *Escherichia coli*, one *Acinetobacter*, and one *Klebsiella pneumoniae*. Salter et al.[34] elucidated that samples with low biomass with minimal microbial content are highly vulnerable to contamination from reagents and environmental factors in the laboratory. In BSIs, the pathogen load may be too low for accurate detection. Utilizing approximately 1 mL of plasma to extract cfDNA for mNGS analysis may result in false-negative outcomes. Comparatively, BC techniques involve a more substantial volume of blood and are advantageous for detecting species such as *E. coli*, which are known for their rapid generation times and amenability to cultivation.

Conclusions

We conducted a prospective clinical study of patients with hematological malignancies to evaluate the diagnostic value of mNGS for BSI. Our findings demonstrate that antibiotic use will greatly reduce the positive rate of mNGS. Even if the detection efficiency of mNGS decreases after the antimicrobial therapy, its duration of positivity is significantly longer than that of conventional BC. Moreover, the duration of mNGS in patients with BSI is associated with the presence of septic shock and could be identified as a high-risk factor for adverse infection outcomes. Therefore, we recommend performing mNGS testing before administering antibiotics to improve the detection rate of pathogenic microorganisms in patients with BSIs. However, performing mNGS testing after antibiotic use is valuable. The sensitivity of mNGS remains higher than that of BC after antibiotic use. Additionally, the duration of positivity for mNGS can assess infection severity and estimate the effectiveness of antibiotic treatment, aiding timely treatment adjustments.

Ethics approval and informed consent

This study was approved by the Medical Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School (IRB No. 2020–377-02) and was conducted in accordance with the Declaration of Helsinki. Patients or their family members were informed of the study in detail and signed informed consent to participate.

Funding

This work was supported by the Key Research & Development Program of Jiangsu Province (BE2023656) and fundings for Clinical Trials from the Affiliated Drum Tower Hospital, Medical School of Nanjing University (2021-LCYJ-MS-19, 2022-LCYJ-PY-46).

Author contributions

All authors contributed substantially to the manuscript and agreed to the final submitted version. Original Draft Writing: Y. Xu and T. Zhou; Review & Editing: P. Xu, B. Chen and J. Ouyang; Clinical Trial Design: Y. Xu, M. Peng, Y. Yang and J. Ouyang; Clinical Trial Management & Data Acquisition: Y. Xu, M. Peng, Y. Yang, T. Xie, X. Cao, P. Xu, B. Chen and J. Ouyang; Data Analysis & Statistics: Y. Xu, M. Peng, T. Zhou and P. Xu; Funding Acquisition: Y. Xu and J. Ouyang; Study Supervision: B. Chen and J. Ouyang.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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