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ORIGINAL RESEARCH

Clinical Utility of Metagenomic Next-Generation Sequencing in Diagnosing Central Nervous System Infections in Hematopoietic Stem Cell Transplant Recipients: A Retrospective and Prospective Cohort Study

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Background: Diagnosing central nervous system infections (CNSI) in hematopoietic stem cell transplant (HSCT) recipients remains challenging due to nonspecific presentations and low sensitivity of conventional microbiological methods.

Methods: This study evaluated the clinical utility of cerebrospinal fluid (CSF) metagenomic next-generation sequencing (mNGS) in 127 HSCT recipients (87 retrospective, 40 prospective) from Peking University People's Hospital. Pathogens detected by mNGS and conventional methods were validated via Sanger sequencing.

Results: mNGS identified 20 pathogen-positive samples (19 confirmed by sequencing), while conventional methods detected none. mNGS demonstrated 82.6% sensitivity and 99.0% specificity for CNSI diagnosis, with sensitivity rising to 100.0% when combined with conventional approaches. Notably, mNGS excelled in detecting viral pathogens, particularly in allogeneic HSCT recipients.

Conclusion: Our findings advocate for the integration of mNGS into the diagnostic algorithm for CNSI, especially in immunocompromised hosts. This approach enables earlier and more precise pathogen identification, which has the potential to streamline antimicrobial therapy and improve clinical management. To maximize its benefit and ensure reliable interpretation, mNGS results should be correlated with comprehensive clinical and paraclinical data. Further prospective studies are warranted to validate its impact on therapeutic decision-making and patient prognosis.

Keywords: central nervous system infection, metagenomic next-generation sequencing, bone marrow transplantation

Background

Central nervous system infections (CNSI), such as encephalitis, myelitis, and meningitis, pose significant diagnostic challenges due to the complexity of their clinical manifestations[1–3] and the limitations of conventional bacterial culture methods, which exhibit a low positive rate of pathogen detection. Despite extensive diagnostic efforts, the etiology remains unidentified in 40%,60% of patients with meningitis or encephalitis.[4] 9% −15% of patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT) suffer from central nervous system (CNS) complications. Although CNSI is considered the main cause, it is difficult to infer the reasons for non-specific clinical manifestations.[5,6] Metagenomic next-generation sequencing (mNGS) of cerebrospinal fluid (CSF) is a hypothesis-free, comprehensive approach for detecting a broad spectrum of infectious agents, including DNA and RNA viruses, bacteria, fungi, and

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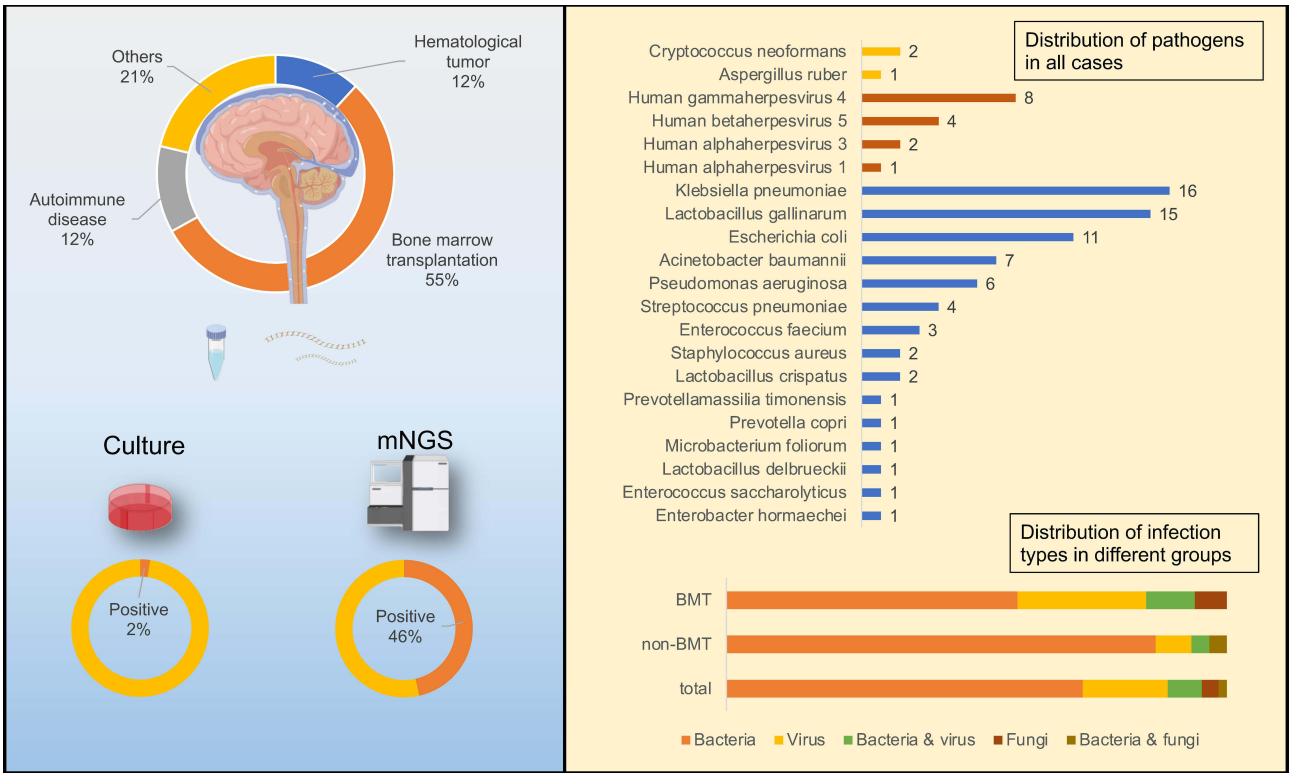
Graphical Abstract



parasites within a single test.[3,7] mNGS is considered a transformative advancement in clinical microbiology, offering advantages such as higher accuracy, an increased positivity rate, a shorter detection time, and reduced susceptibility to prior antimicrobial use compared to conventional microbiological detection methods.[8] However, its high sensitivity also presents challenges, including the potential for contamination and false-positive results, which necessitate careful interpretation and validation.[9] Studies utilizing mNGS have demonstrated that the spectrum of pathogens varies across different populations with CNSI.[4,10–12] Given the presence of a large-scale bone marrow transplantation (BMT) center, we investigated the use of mNGS in patients with suspected CNS infection who had undergone allogeneic hematopoietic stem cell transplantation (allo-HSCT). Previous research indicated that among post-BMT patients, the survival rate is 80% in those without CNS infection, whereas it declines significantly to 26.7% in those with CNSI complications.[6] Previous studies have demonstrated that mNGS of CSF enhances the diagnosis of neurological infections, providing actionable clinical insights in some cases.[13]

To evaluate the clinical utility of mNGS in diagnosing CNSI, we enrolled both retrospective and prospective patient cohorts. We assessed the sensitivity and specificity of mNGS in identifying pathogens in CSF, with results confirmed by conventional microbiological methods and Sanger sequencing. This study was designed to assess the real-world clinical performance of mNGS and explore its potential for routine diagnostic application.

Materials and Methods

Ethics Statement

This study was reviewed and approved by the Peking University People's Hospital Institutional Review Board (approval nos. 2022PHA009-001 and 2022PHA008-001). All procedures adhered strictly to the Regulations of the Ethical Review of Biomedical Research Involving Human Subjects (2016), Declaration of Helsinki, and International Ethical Guidelines for Biomedical Research Involving Human Subjects.[14]

Patients and Samples

A total of 127 patients with suspected CNSIs were prospectively enrolled for this study between May 2018 and August 2022. The suspicion of CNS infections was based on the presence of acute fever (> 38.5°C) accompanied by at least one of the following clinical signs: (1) stiff neck, (2) altered consciousness, (3) increased anterior fontanelle tension, or (4) meningeal irritation.[15] After obtaining informed consent, CSF samples were collected for pathogen detection using mNGS, Sanger sequencing, and conventional diagnostic methods, which included microbial culture, ink staining, and cryptococcal capsular antigen (CrAg) detection. Study design is shown in Supplementary Figure 1. The clinical characteristics and laboratory findings of the enrolled patients, along with corresponding conventional diagnostic results, were retrieved from the Hospital Information System of Peking University People's Hospital.

mNGS

CSF samples were collected into sealed sterile tubes and temporarily stored at temperatures below −70°C. As previously described,[16] we established a microbial mNGS and data analysis platform for diagnostic purposes, including DNA extraction, library construction, sequencing, and pathogen identification. Briefly, DNA was extracted from CSF samples using the QIAamp DNA Microbiome Kit (Qiagen). DNA concentrations were quantified using the Qubit dsDNA high sensitivity (HS) Assay Kit (Thermo Fisher Scientific), with sterile deionized water processed alongside the specimens as a negative control. Library preparation and sequences were performed using the NextSeq 550Dx High Output Reagent Kit v2.5 (Illumina). A 75-bp single-end sequencing was performed and at least 20 million sequencing reads were obtained for each sample.

Following adapter trimming and the removal of low-quality and low-complexity reads, clean reads were subjected to microbial identification. The reference genome database included human (*hg38*), along with185630bacterial, 308 archaeal, 54 fungal, 9021 viral, 178 invertebrate, and 39 protozoans genomes, all downloaded from the National Center for Biotechnology Information. Reference databases were created, and taxonomy assignment was performed using Kraken2 v2.0.8beta.[17] After alignment to the reference genome, pathogens of potential clinical significance were defined based on the following criteria: (1) 100% full-length alignment to a microbial genome, (2) sequence similarity exceeding 95%, and (3) identification of a unique microbial genome meeting the above alignment conditions.

We developed threshold criteria for determining pathogen positivity to reduce false-positive results caused by microbial contamination as previous described.[18] The no-template control (NTC) was sterile water that ran in parallel with the clinical test samples for each batch to monitor contamination during the mNGS process. The positive identification criteria for bacterial, fungal, and parasitic pathogens were defined by a species-specific read-per-million (RPM) ratio (Rr), calculated as follows: $Rr = (RPM_{sample}+0.1)/(RPM_{blank}+0.1)$. Cutoff values of Rr were determined using receiver operating characteristic (ROC) curve analysis (Supplemental Table 1).

Sanger Sequencing

DNA extraction and purification were performed using the QIAamp DNA Microbiome Kit (Qiagen) according to the manufacturer's instructions. The extracted DNA was then amplified by polymerase chain reaction (PCR) using specific primers (Supplemental Table 2) and Premix Taq^{TM} (Takara). PCR products were electrophoresed on a 2% agarose gel, and the target DNA fragment of the expected length was extracted for sequencing. Two-way Sanger sequencing was performed using a BigDye Terminator V3.1 (Applied Biosystems). The resulting sequences were aligned and analyzed using the Basic Local Alignment Search Tool (BLAST) to determine pathogen identity.

Statistical Analysis

Data were assessed for normality using the Kolmogorov–Smirnov test in GraphPad Prism (GraphPad Software Inc., California, USA). Continuous variables were presented as medians with interquartile ranges(IQRs) for non-normally distributed data, while categorical variables were reported as numbers (percentages) of the total sample. Mann–Whitney *U*-test was performed for non-normally distributed data and *t*-test was for normally distributed data. The $\chi 2$ test or Fisher exact test was utilized for comparison of categorical variables. $P < 0.05$ was considered statistically significant. ROC

curves were generated using GraphPad Prism (GraphPad Software Inc., California, USA). Statistical analyses were conducted using SPSS version 26.0 (IBM Corporation, Armonk, NY, USA). Principal Component Analysis (PCA) was performed using R software (version 4.2.2).

Results

Patient Characteristics

A total of 127 patients were enrolled in this study, comprising two cohorts: retrospective and prospective (Table 1). The retrospective cohort consisted of 51 males and 36 females, with a median age of 30 years (Interquartile Range [IQR]: 21,51 years), while the prospective cohort included 20 males and 20 females, with a median age of 44 years (IQR: 24,73 years) (Table 1). Common symptoms among all enrolled patients included fever (29.9%), headache (22.0%), hyperpasmia (11.8%), loss of consciousness (11.8%), and weakness in the arms and legs (14.2%) (Table 1). Nearly half of the CSF samples (49.6%) were collected within a week of symptom onset (Table 1). The distribution of underlying disease varied significantly between the two cohorts ($p < 0.001$), with allo-HSCT being the predominant condition in the retrospective cohort (64.4%) (Table 1). Penicillin and meropenem were the most frequently administered antibiotics, accounting for 18.1% and 18.9% of cases, respectively (Table 1).

Table 1 Characteristics of Patients Enrolled in This Study

	Retrospective Cohort (n = 87)	Prospective Cohort (n = 40)	Total (n = 127)	p value*
Gender				0.363
Male	51 (58.6)	20 (50.0)	71 (55.9)	
Female	36 (41.4)	20 (50.0)	56 (44.1)	
Age (years, median, IQR)	30 (21, 51)	44 (24, 73)	34 (21, 53)	0.056
Onset of symptoms/signs				0.145
Fever	25 (28.7)	13 (32.5)	38 (29.9)	
Headache	24 (27.6)	4 (10.0)	28 (22.0)	
Hyperspasmia	12 (13.8)	3 (7.5)	15 (11.8)	
Loss of consciousness	10 (11.5)	5 (12.5)	15 (11.8)	
Weakness in the arms/legs	8 (9.2)	10 (25.0)	18 (14.2)	
Nausea	7 (8.0)	0 (0.0)	7 (5.5)	
Dizziness	6 (6.9)	2 (5.0)	8 (6.3)	
Blurred vision	5 (5.7)	0 (0.0)	5 (3.9)	
Mental disorder	5 (5.7)	3 (7.5)	8 (6.3)	
Epilepsy	3 (3.4)	1 (2.5)	4 (3.1)	
Tremor	2 (2.3)	0 (0.0)	2 (1.6)	
Speech impairment	2 (2.3)	0 (0.0)	2 (1.6)	
Angular deviation	1 (1.1)	0 (0.0)	1 (0.8)	
Intracranial hypertension	1 (1.1)	0 (0.0)	1 (0.8)	
Dysphagia	1 (1.1)	0 (0.0)	1 (0.8)	
Inadequate closure of eyelids	1 (1.1)	0 (0.0)	1 (0.8)	
Abnormal MRI	0 (0.0)	1 (2.5)	1 (0.8)	
Duration of Symptoms				0.148
Less than 1 week	45 (51.7)	18 (45.0)	63 (49.6)	
1-2 weeks	14 (16.1)	4 (10.0)	18 (14.2)	
2 weeks to 3 months	14 (16.1)	12 (30.0)	26 (20.5)	
More than 3 months	3 (3.4)	4 (10.0)	7 (5.5)	
Underlying disease				<0.001
Allo-HSCT	56 (64.4)	14 (35.0)	70 (55.1)	
Hematologic malignancies	13 (14.9)	2 (5.0)	15 (11.8)	

(*Continued*)

Table 1 (Continued).

	Retrospective Cohort (n = 87)	Prospective Cohort (n = 40)	Total (n = 127)	p value*
Autoimmune disease	5 (5.7)	6 (15.0)	11 (8.7)	
Cerebrovascular disease	2 (2.3)	3 (7.5)	5 (4.0)	
Guillain-Barre Syndrome	1 (1.1)	2 (5.0)	3 (2.4)	
Others	1 (1.1)	6 (15.0)	7 (5.5)	
Antibiotic treatment				0.564
Penicillins	20 (23.0)	3 (7.5)	23 (18.1)	
Meropenem	19 (21.8)	5 (12.5)	24 (18.9)	
Aminoglycosides	8 (9.2)	2 (5.0)	10 (7.9)	
Quinolones	7 (8.0)	3 (7.5)	10 (7.9)	
Third/fourth generation cephalosporins	6 (6.9)	2 (5.0)	8 (6.3)	
Vancomycin	6 (6.9)	0 (0.0)	6 (4.7)	
Nitroimidazoles	2 (2.3)	2 (5.0)	4 (3.1)	
Sulfonamides	2 (2.3)	0 (0.0)	2 (1.6)	

Notes: * p value was calculated between retrospective and prospective cohorts, using statistical methods of Chi-square tests. p <0.05 was seen as significant.
Abbreviations: IQR, Inter-Quartile Range; Allo-HSCT, allogeneic hematopoietic stem cell transplantation.

Pathogen Distribution Across Different Detection Methods in CSF

We collected pathogen detection results from conventional methods, including culture, ink staining, and CrAg detection, along with corresponding findings from Sanger sequencing and mNGS. In the retrospective cohort, *Corynebacterium minutissimum* (*C. minutissimum*) and *Staphylococcus lugdunensis (S. lugdunensis)* were detected via culture, whereas *Rosemonas marceus (R. marceus)* was identified in the prospective cohort (Table 2, Figure 1A). All other samples were negative for ink staining, and only one sample in the retrospective cohort tested positive for CrAg (Table 2, Figure 1A).

Table 2 Pathogens Distribution of Different Detection Methods in CSF

	Retrospective Cohort (n = 87)	Prospective Cohort (n = 40)	Total (n = 127)	p value*
Conventional methods				
Culture				
Negative	85 (97.7)	31 (77.5)	116 (91.3)	
R. marceus	0 (0.0)	1 (2.5)	1 (0.8)	
C. minutissimum	1 (1.1)	0 (0.0)	1 (0.8)	
S. lugdunensis	1 (1.1)	0 (0.0)	1 (0.8)	
Not performed	0 (0.0)	8 (20.0)	8 (6.3)	
Ink stain				
Negative	85 (97.7)	26 (65.0)	111 (87.4)	
Not performed	2 (2.3)	14 (35.0)	16 (12.6)	
CrAg				
Positive	1 (1.1)	0 (0.0)	1 (0.8)	
Negative	81 (93.1)	28 (70.0)	109 (85.8)	
Not performed	5 (5.7)	12 (30.0)	17 (13.4)	
Sanger sequencing				0.843
C. neoformans	2 (2.3)	0 (0.0)	2 (1.6)	
CMV	1 (1.1)	1 (2.5)	2 (1.6)	
EBV	6 (6.9)	1 (2.5)	7 (5.5)	

(*Continued*)

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Table 2 (Continued).

	Retrospective Cohort (n = 87)	Prospective Cohort (n = 40)	Total (n = 127)	p value*
EBV & CMV	1 (1.1)	0 (0.0)	1 (0.8)	
HSV-1	1 (1.1)	0 (0.0)	1 (0.8)	
VZV	1 (1.1)	1 (2.5)	2 (1.6)	
S. pneumoniae	1 (1.1)	0 (0.0)	1 (0.8)	
S. aureus	1 (1.1)	0 (0.0)	1 (0.8)	
E. coli	1 (1.1)	0 (0.0)	1 (0.8)	
K. pneumoniae	1 (1.1)	0 (0.0)	1 (0.8)	
Negative	71 (81.6)	37 (92.5)	108 (85.0)	
mNGS				0.861
C. neoformans	2 (2.3)	0 (0.0)	2 (1.6)	
CMV	2 (2.3)	1 (2.5)	3 (2.4)	
EBV	6 (6.9)	1 (2.5)	7 (5.5)	
EBV & CMV	1 (1.1)	0 (0.0)	1 (0.8)	
HSV-1	1 (1.1)	0 (0.0)	1 (0.8)	
VZV	1 (1.1)	1 (2.5)	2 (1.6)	
S. pneumoniae	1 (1.1)	0 (0.0)	1 (0.8)	
S. aureus	1 (1.1)	0 (0.0)	1 (0.8)	
E. coli	1 (1.1)	0 (0.0)	1 (0.8)	
K. pneumoniae	1 (1.1)	0 (0.0)	1 (0.8)	
Negative	70 (80.5)	37 (92.5)	107 (84.3)	

Notes: * p was calculated between retrospective and prospective cohorts, using statistics methods of Chi-square tests. p <0.05 was considered as significant.
Abbreviations: *R. marceus*, *Rosemonas marceus*; *C. minutissimum*, *Corynebacterium minutissimum*; *S. lugdunensis*, *Staphylococcus lugdunensis*; CrAg, cryptococcal capsular antigen detection.

A total of nine species were identified by Sanger sequencing and mNGS, including *C. neoformans*, human cytomegalovirus (CMV), *Epstein-Barr virus* (EBV), Herpes simplex virus type I (HSV- I), *Varicella zoster virus* (VZV), *Streptococcus pneumoniae (S. pneumoniae), Staphylococcus aureus (S. aureus), Escherichia coli (E. coli)* and *Klebsiella pneumoniae (K. pneumoniae)* (Table 2, Figure 1A). Compared to Sanger sequencing, mNGS detected an additional CMV-positive sample that was not identified by Sanger sequencing. Notably, all pathogen-positive samples detected by either Sanger sequencing or mNGS were reported as negative by conventional methods (Figure 1A and B). In addition, we collected laboratory data from CSF samples, such as erythrocyte counts, leukocyte counts, and biochemical parameters, including total protein, glucose, and chloride ions (Cl^-). Based on results from conventional methods and Sanger sequencing, the CSF samples were separated into four groups according to the detected pathogens type: fungal, bacterial, viral, and negative. However, distinguishing infection types based solely on cell counts and biochemical examination results remained challenging (Supplemental Figure 2A–D).

Performance of mNGS in the Diagnosis of Pathogens in CSF

To evaluate the diagnostic performance of mNGS for pathogen detection in CSF, we established a reference criterion, defining a positive result as any pathogen identified by conventional methods or Sanger sequencing. As illustrated by the ROC curves, the area under the curve (AUC) for mNGS was 0.908, while Sanger sequencing achieved an AUC of 0.913, and conventional methods had a lower AUC of 0.565 (Figure 2A–C). When mNGS was combined with conventional methods, the AUC increased to 0.996 (Figure 2D). The specificity and sensitivity of mNGS were 99.0% and 82.6%, respectively (Table 3). Conventional methods and Sanger sequencing both demonstrated a specificity of 100.0%, while their sensitivities were 17.4% and 82.6%, respectively (Table 3). Notably, combining mNGS with conventional methods increased sensitivity to 100.0%, while specificity remained at 99.0%. Furthermore, the positive predicted value (PPV)

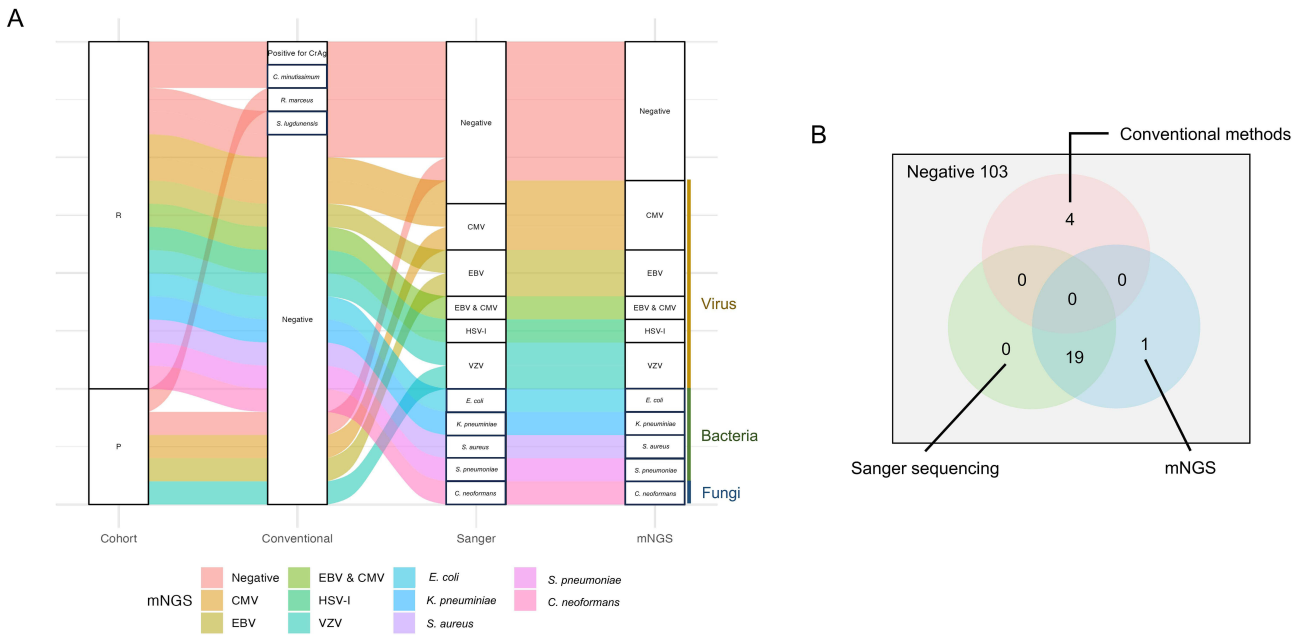

Figure 1 Agreement of pathogen detection methods in cerebrospinal fluid (CSF). (**A**) Pathogens detected by conventional methods, Sanger sequencing and Metagenomic next generation sequencing (mNGS); (**B**) Performance of conventional methods, Sanger sequencing and mNGS. Conventional methods included culture, ink stain, and cryptococcal capsular antigen detection (CrAg). Positive result of conventional methods was considered if there was a positive result from any of the three methods.
Abbreviations: P, prospective cohort; R, retrospective cohort; C. neoformans, Cryptococcus neoformans; CMV, human cytomegalovirus; EBV, *Epstein-Barr virus*; HSV-I, Herpes simplex virus type I; VZV, *Varicella zoster virus*; *S. pneumoniae*, *Streptococcus pneumoniae*; *S. aureus*, *Staphylococcus aureus*; *E. coli*, *Escherichia coli*; *K. pneumoniae*, *Klebsiella pneumoniae*.

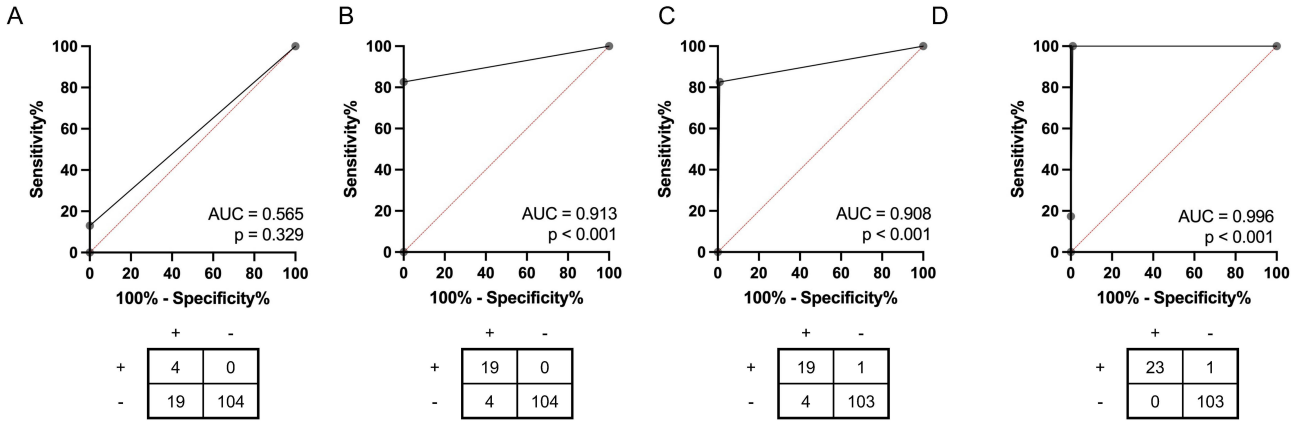

Figure 2 Receiver operating characteristic curves (ROC) of different methods in pathogens of CSF. (**A**) Conventional methods; (**B**) Sanger sequencing; (**C**) mNGS; (**D**) Combined mNGS and conventional methods. The diagnostic criteria was set as a positive result from conventional methods or Sanger sequencing. The column of fourfold table showed the results met with diagnostic criteria and the row showed the related result of methods.
Abbreviation: AUC, area under curve.

reached 95.8% and negative predicted value (NPV) reached 100%, exceeding the predictive accuracy of any single diagnostic approach (Table 3).

Pathogens Detected in Patients with Different Underlying Diseases

Pathogens were most frequently detected in patients who had undergone allo-HSCT (14/20), followed by those with underlying hematological malignancies (HM) (3/20) (Figure 3A). In contrast, only *C. minutissimum* and *R. marceus* were detected using conventional methods in the allo-HSCT and HM groups, respectively (Figure 3B). Among patients who underwent allo-HSCT, EBV was most frequently detected (6/14), followed by CMV (2/14), with one patient co-infected

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Table 3 Diagnostic Performance of Different Detection Methods in CSF

	Conventional Methods	Sanger Sequencing	mNGS	Combined mNGS and Conventional Methods
Positive, n (%)	4 (3.1)	19 (15.0)	20 (15.7)	24 (18.9)
Negative, n (%)	123 (96.9)	108 (85.0)	107 (84.3)	103 (81.1)
Sensitivity (%, 95% CI)	17.4 (0.6–39.5)	82.6 (60.5–94.3)	82.6 (60.5–94.3)	100.0 (82.2–100.0)
Specificity (%, 95% CI)	100.0 (95.6–100.0)	100.0 (95.6–100.0)	99.0 (94.0–99.9)	99.0 (94.0–99.9)
PPV (%, 95% CI)	100.0 (39.6–100.0)	100.0 (79.1–100.0)	95.0 (73.1–99.7)	95.8 (76.9–99.8)
NPV (%, 95% CI)	84.5 (76.7–90.2)	96.3 (90.2–98.8)	96.3 (90.1–98.8)	100.0 (95.5–100.0)

Abbreviations: PPV, positive predictive value; NPV, negative predictive value; CI, confidence intervals.

with both EBV and CMV (Figure 3A). Additionally, *C. neoformans*, HSV- I, VZV, *S. aureus*, and *E. coli* were each detected once in patients who underwent allo-HSCT. In the HM group, *K. pneumoniae, C. neoformans*, and CMV were each detected in one patient (Figure 3A). We also analyzed relative pathogen abundance by grouping all RPM values according to disease category. Analysis revealed distinct patterns of microbial relative abundance: *S. aureus*, CMV, EBV, and *E. coli* were most prominent in the allo-HSCT group, *K. pneumoniae* in the HM group, and VZV among viral infections, as indicated by their respective RPM values (Figure 3C).

Discussion

Metagenomic next-generation sequencing has been increasingly used for the diagnosis of lower respiratory tract infections (LRTI),[16] fever of unknown origin (FUO),[19] bloodstream infections (BI)[20] and CNSI.[12] Opportunistic CNSI, particularly viral encephalitis, was common among patients undergoing bone marrow transplantation.[6,21,22] Due

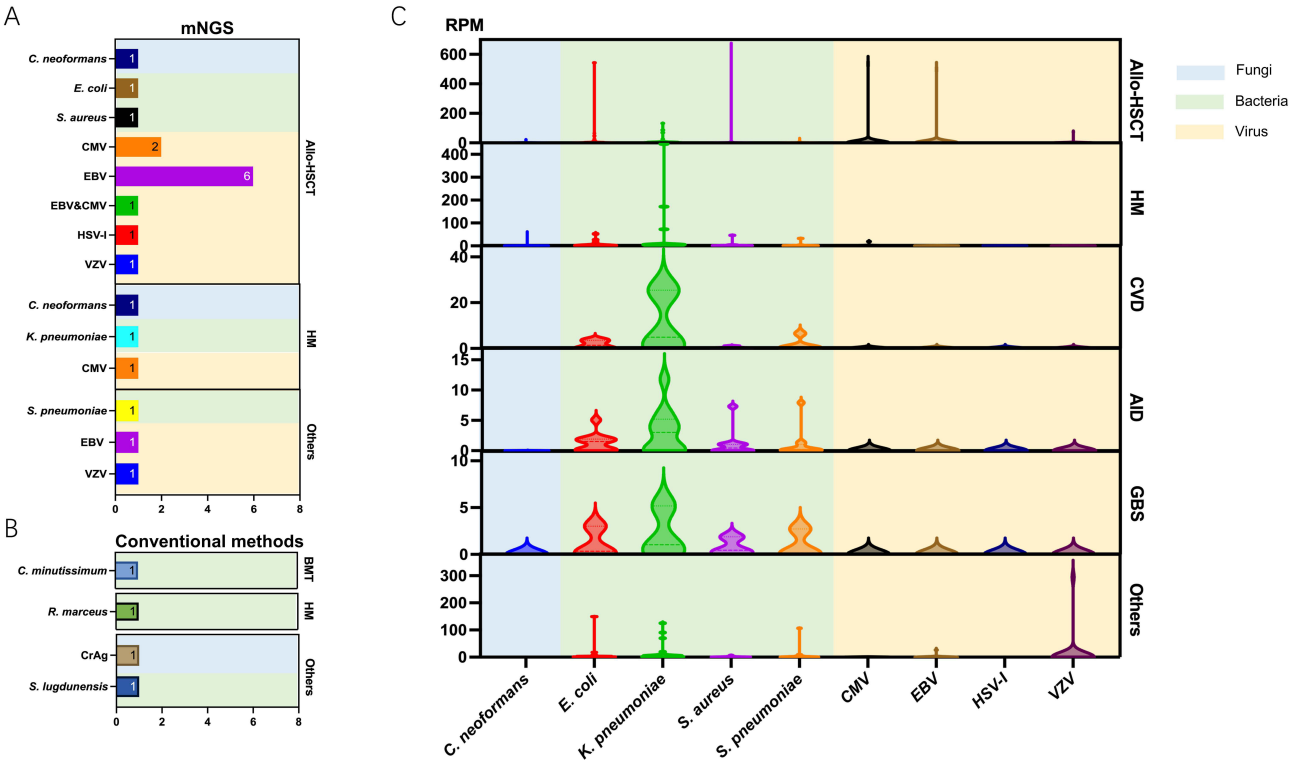

Figure 3 Pathogens detected in patients with different underlying disease. (**A**) Pathogens detected by mNGS; (**B**) Pathogens detected by conventional methods; (**C**) Reads per million (RPM) of pathogens detected by mNGS.

Abbreviations: Allo-HSCT, allogeneic hematopoietic stem cell transplantation; HM, Hematologic malignancies; CVD, Cerebrovascular disease; AID, autoimmune disease; GBS, Guillain-Barre Syndrome.

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to the limitations of conventional diagnostic methods in diagnosing CNSI, most cases of viral encephalitis were not routinely identified during clinical evaluations. Our study evaluated the clinical utility of mNGS in diagnosing CNSI among patients with suspected symptoms, including those who had undergone gallo-HSCT, had HM, autoimmune diseases, Guillain-Barré syndrome, cardiovascular diseases, or other neurological conditions. Remarkably, mNGS detected 20 pathogen-positive cases, whereas conventional methods identified only four. Furthermore, 19 of the mNGS-positive cases were confirmed by Sanger sequencing. When combined with conventional methods, mNGS achieved a diagnostic sensitivity and specificity of 100.0% and 99.0%, respectively. These findings provide strong evidence that the routine application of mNGS can address the limitations of conventional diagnostic methods and serve as a valuable tool for identifying CNSI pathogens.[23]

Previous studies have reported that mNGS has a sensitivity range of 73,92% and a specificity of 96,99%, depending on the pathogen detected[3,24] In our study, mNGS demonstrated a sensitivity of 82.6% and a specificity of 99.0%, aligning with previous research. Notably, combining mNGS with conventional methods further increased sensitivity to 100.0%, underscoring the advantages of integrating molecular diagnostics with conventional microbiological approaches. We also enrolled two cohorts to estimate the role of mNGS in diagnosing CSF pathogens: a retrospective and prospective cohort. Although the disease distribution differed significantly between the cohorts, mNGS successfully detected pathogens that were not identified by conventional methods in both cohorts. Wilson et al retrospectively investigated the etiology of chronic meningitis using mNGS and successfully identified diagnostically challenging pathogens in the CSF of patients with subacute or chronic meningitis.[25]

In our study, due to the specialized patient population at our center, 55.1% of enrolled patients had undergone allo-HSCT. As a result of preconditioning regimens associated with the transplantation procedure and empirical antibiotic therapy (details in Supplemental methods), the etiology of most fever cases following alloHSCT remained undetermined. Additionally, 9%,15% of patients post-allo-HSCT experience CNS complications, which present with non-specific symptoms such as disturbances of consciousness, convulsions, headaches, fever, and epilepsy. When CSF pathogen testing yields negative results using conventional methods, determining the underlying etiology remains a significant challenge.[6] A growing body of evidence suggests that viruses have become the predominant cause of CNSI after HSCT.[13,26–28] Among these, EBV is a frequently detected virus in patients with CNS complications after allo-HSCT and is associated with a high risk of mortality.[6,29,30] In our study, EBV was the most frequently detected virus in the CSF of patients who had undergone allo-HSCT (6/14), followed by CMV (2/14). Notably, none of these patients exhibited EBV viremia, and only one case of CMV-related CNSI was identified. Interestingly, mNGS detected only one case of CMV-CNSI, whereas cytomegalovirus was identified in 19 enrolled patients. Additionally, two *C. neoformans* isolates were detected by mNGS, one from a patient with HM and one from a patient post-allo-HSCT, while neither was detected by conventional methods (Supplemental Table 3). These findings highlight a key advantage of mNGS: its hypothesis-free approach allows for the detection of multiple potential pathogens in a single assay without the need for a priori selection of targeted pathogens.[13,31]

Despite the high sensitivity of mNGS for pathogen detection, false-negative results were observed in our study. Three samples that tested positive by culture for *S. lugdunensis, C. minutissimum*, and *R. marceu*, as well as one CrAg-positive sample from a patient with severe CNSI symptoms, were not detected by mNGS (Supplemental Figure 2) or Sanger sequencing. We considered that culture, when unaffected by prior antibacterial or antifungal therapy, might have a lower detection limit than that of mNGS and Sanger sequencing for certain easily cultured pathogens. The four false-negative cases could be attributed to several plausible factors we will now discuss, such as: extremely low pathogen load below the detection limit, suboptimal sample volume or quality, technical limitations in nucleic acid extraction or bioinformatic analysis for certain pathogens, or intracellular sequestration of the pathogen.[18] Therefore, we integrated mNGS with conventional methods to enhance diagnostic accuracy. As reported in previous studies, the median time to result for mNGS is approximately 38 hours, which is significantly shorter than that of Sanger sequencing.[32] Considering that mNGS data are now increasingly affordable and accessible, we recommend its regular use of mNGS in conjunction with conventional methods, particularly in settings encountering chronic or recurring infections and immunosuppressive populations. The integration of mNGS in these cases can facilitate the identification of injection origins in complex clinical cases with incomplete diagnostic information.[17]

Our study was limited by a non-random patient selection method and a relatively small sample size. Due to the high sensitivity of mNGS, some results may represent false positives, as they were not verified through additional diagnostic methods such as brain magnetic resonance imaging (MRI). In addition, four cases with false-negative results were observed, the underlying cause of which remained unknown. In the interpretation of our data, potential sources of false-positive signals must be considered, including contamination from laboratory engineering strains, cross-contamination during experimental procedures, inaccuracies within taxonomic reference databases, and index hopping during sequencing.[18] Besides, the definition of a "positive result" for the ROC analysis relies heavily on Sanger sequencing, which itself may have limitations. In the future, more accurate diagnostic criteria should be developed for CNSI, especially for patients after HSCT. To advance the clinical application of mNGS, future efforts should follow a structured path. First, a diagnostic composite reference standard integrating multifaceted clinical data must be established. Second, rigorous multi-angle validation of mNGS results—encompassing orthogonal technical confirmation, host-response profiling, and clinical correlation—is essential to distinguish true infections. Finally, the ultimate utility must be evaluated through prospective studies assessing the impact of mNGS on therapeutic decision-making and patient outcomes.

Conclusions

In conclusion, our evaluation supports the significant additive value of mNGS in the diagnostic workflow for CNS infections, particularly within complex, immunocompromised populations such as allo-HSCT recipients. By successfully identifying pathogens missed by conventional methods—most notably latent viruses and fastidious organisms—this study underscores the critical role of a comprehensive, hypothesis-free diagnostic approach in etiologically elusive cases. The integration of mNGS with conventional microbiology can effectively enhance diagnostic sensitivity, promising to reduce empirical therapy and enable more targeted interventions. However, the clinical interpretation of its high-sensitivity results necessitates caution and should be corroborated within a multi-parameter clinical context. To realize its full potential, future work must focus on standardizing diagnostic criteria, validating results through orthogonal clinical and laboratory frameworks, and ultimately demonstrating improved patient outcomes through prospective interventional studies. This work thus contributes a pragmatic step towards precision infectious disease diagnostics in high-risk neurology practice.

Abbreviations

AUC, Area Under the Curve; BI, Bloodstream Infection; BLAST, Basic Local Alignment Search Tool; BMT, Bone Marrow Transplantation; CNSI, Central Nervous System Infection; CMV, Cytomegalovirus; CrAg, Cryptococcal Capsular Antigen; CSF, Cerebrospinal Fluid; EBV, *Epstein-Barr virus*; FUO, Fever of Unknown Origin; GBS, Guillain-Barré Syndrome; HM, Hematological Malignancy; HSCT, Hematopoietic Stem Cell Transplantation; IQR, Interquartile Range; LRTI, Lower Respiratory Tract Infection; mNGS, Metagenomic Next-Generation Sequencing; MRI, Magnetic Resonance Imaging; NPV, Negative Predictive Value; PCA, Principal Component Analysis; PPV, Positive Predictive Value; Rr, Read-Per-Million Ratio; ROC, Receiver Operating Characteristic; *S. aureus, Staphylococcus aureus; S. lugdunensis, Staphylococcus lugdunensis; S. pneumoniae, Streptococcus pneumoniae*; SPSS, Statistical Package for the Social Sciences; VZV, Varicella-Zoster Virus.

Data Sharing Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study was reviewed and approved by the Peking University People's Hospital Institutional Review Board (approval nos. 2022PHA009-001 and 2022PHA008-001). This study was approved by the research ethics board at Peking University People's Hospital (ID: 2019PHB134).

All procedures adhered strictly to the Regulations of the Ethical Review of Biomedical Research Involving Human Subjects (2016), Declaration of Helsinki, and International Ethical Guidelines for Biomedical Research Involving Human Subjects.

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Author Contributions

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

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Disclosure

The authors declare that they have no competing interests.

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