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An Investigation into Diagnostic Strategies for Central Nervous System Infections Through the Integration of Metagenomic Next-Generation Sequencing and Conventional Diagnostic Methods

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Purpose: The optimal strategy for detecting central nervous system infections (CNSI) in cerebrospinal fluid (CSF) samples remains unclear.

Methods: In a one-year, multicenter retrospective study, we examined the efficacy of metagenomic next-generation sequencing (mNGS) in comparison to conventional pathogen diagnostic techniques for CSF in diagnosing CNSI. We calculated the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and Youden index for each diagnostic approach. Additionally, receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) was determined to assess the diagnostic performance of each method.

Results: The study included 68 patients, comprising both adults and children, who were suspected of having CNSI. Through the application of comprehensive clinical interpretation (CCI), the sensitivity and specificity of mNGS were found to be 67.6% (95% confidence interval [CI]: 50.85–80.87%) and 45.8% (95% CI: 27.89–64.92%), respectively. In comparison, traditional pathogenic diagnostic methods indicated that the culture method demonstrated a sensitivity of 10.6% (95% CI: 4.63–22.6%) and a specificity of 100% (95% CI: 84.54–100%). Furthermore, the sensitivity and specificity of the peripheral blood nucleated cell count were determined to be 34.0% (95% confidence interval: 22.17–48.33%) and 57.1% (95% confidence interval: 36.54–75.53%), respectively. CSF nucleated cell count demonstrated a sensitivity of 66.0% (95% confidence interval [CI]: 51.67–77.83%) and a specificity of 61.9% (95% CI: 40.87–79.25%). In comparison, the CSF protein content exhibited a sensitivity of 63.8% (95% CI: 49.54–76.03%) and a specificity of 57.1% (95% CI: 36.54–75.53%). When combining mNGS with traditional methodologies, the overall sensitivity increased to 91.3% (95% CI: 79.67–96.56%), although the specificity was reduced to 18.2% (95% CI: 7.31–38.51%). The area under the ROC curve for culture, peripheral blood nucleated cell count, mNGS, CSF nucleated cell count, and CSF protein content were 0.8088, 0.6038, 0.6103, 0.5588, and 0.5588, respectively. The variation in CSF nucleated cell count did not significantly affect the diagnostic efficacy of mNGS.

Conclusion: Currently, both mNGS and traditional diagnostic methods encounter substantial challenges in diagnosing CNSI.

Keywords: mNGS, CSF, central nervous system infections, ROC

Introduction

Central nervous system infections (CNSI) encompass conditions such as encephalitis, meningitis, and myelitis, which may be attributed to bacterial, fungal, viral, or parasitic etiologies. The clinical presentation of infections caused by diverse pathogens often lacks distinctive features, rendering the diagnosis of CNSI particularly challenging.[1] Traditional diagnostic methodologies for pathogen identification include smear microscopy, culture, polymerase chain reaction

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(PCR), antigen-antibody detection, and various auxiliary examination techniques. Notably, the efficacy of culture-based methods can be significantly compromised if the patient has received antibiotic treatment prior to sample collection. The administration of antibiotics prior to sample collection, along with inadequate sample volume, may diminish the positive yield of culture-based methods. The sensitivity of smear microscopy is limited and significantly influenced by pathogen concentration. The PCR technique exhibits high sensitivity and specificity; however, its application is restricted to the targeted screening of individual pathogens. Accurately predicting pathogens imposes substantial challenges on clinicians.

Auxiliary diagnostic indicators encompassed peripheral blood nucleated cell count, CSF nucleated cell count, and CSF levels of protein, glucose, chloride, lactate, among other tests. While the specificity of these indicators for CNSI was limited, integrating these common auxiliary indicators with traditional diagnostic techniques may yield valuable insights. Developing a predictive model based on routine physical and chemical examination indicators of CSF, such as protein levels and nucleated cell count, among others, held potential reference value for the diagnosis of CNSI.[2,3]

As an innovative molecular biological detection method, mNGS technology addresses certain limitations inherent in traditional diagnostic techniques for infectious disease identification.[4] By employing a design principle centered on non-targeted amplification, mNGS technology was capable of detecting pathogens beyond the scope of conventional diagnostic methods.[5] Simultaneously, this approach enables the concurrent detection of bacteria, viruses, fungi, parasites, and atypical pathogens, offering significant advantages in diagnosing multiple infections.[6] However, the complexity inherent in the design and operational processes of non-targeted amplification presents substantial challenges, such as contamination and background interference, which complicate the interpretation of the results.[7] The accurate identification of pathogens with low read numbers presents a significant challenge in the interpretation of mNGS reports.[8] In comparison to traditional culture methods, mNGS exhibits a higher rate of false positives in pathogen detection. Consequently, the clinical application of mNGS in diagnosing and treating infectious diseases necessitates ongoing data accumulation for continuous evaluation. Currently, mNGS is extensively utilized in the diagnosis of infectious diseases, particularly demonstrating notable superiority over traditional diagnostic techniques in cases of lower respiratory tract infections.[8–10] However, there remains a paucity of literature comparing the diagnostic performance of mNGS with traditional pathogenic diagnostic techniques in CNSI.[11]

Materials and Methods

Study Design and Participants

This study was a multi-center retrospective analysis involving patients suspected of CNSI from three geographically distinct branches of Tongji Hospital. The clinical correlation between the results of mNGS and traditional diagnostic techniques was assessed based on clinical comprehensive information (CCI). The determination of whether mNGS results were associated with CNSI was made through collaborative discussions between the clinical physician team and the laboratory team. The patient's gender, age, underlying medical conditions, and additional relevant information can be accessed through electronic medical records. The inclusion criteria for patient selection are as follows: (1) clinical manifestations of CNSI, such as a body temperature exceeding 38.0°C, headache, neck stiffness, among others; (2) alterations in mental status, including symptoms like drowsiness and irritability; (3) CSF pleocytosis greater than 5/mm^3; (4) neuroimaging findings indicative of encephalitis or meningitis.[5]

This retrospective study adhered to the principles outlined in the Declaration of Helsinki and received approval from the Ethics Committee of Tongji Hospital, affiliated with Tongji Medical College of Huazhong University of Science and Technology. Given the retrospective nature of the study design, the ethics committee granted an exemption from obtaining informed consent from the participating patients, ensuring that all clinical information was anonymized prior to analysis.

Collection and Transportation of Samples

CSF samples were acquired via lumbar puncture and collected in sterile storage tubes. The initial tube was designated for biochemical analyses, such as protein quantification. The second tube was allocated for microbiological assessments, including cultivation and Gram staining. The third tube was reserved for cell counting procedures. The fourth CSF

sample was allocated for mNGS analysis. It is imperative that CSF samples are promptly transported to the laboratory for testing immediately following collection. The initial CSF sample was designated for biochemical analysis. In instances where immediate testing is not feasible, the sample should be preserved at −20°C. The sample intended for microbial cultivation, contained in the second tube, should be maintained at ambient temperature. Additionally, the fourth tube sample designated for mNGS detection requires refrigeration.

Diagnostic Criteria for CNSI

The diagnosis of CNSI encompassed both pathogen-based and clinical diagnostic approaches. Individuals who satisfied criteria 1 through 5 were classified under pathogen diagnosis, while those meeting criteria 1 through 4 were categorized under clinical diagnosis.[12]

(1) Clinical manifestations include: ① alterations in consciousness and mental state; ② symptoms indicative of elevated intracranial pressure; ③ signs of meningeal irritation; ④ accompanying symptoms such as epilepsy and hyponatremia; ⑤ symptoms of systemic infection, characterized by manifestations of a systemic inflammatory response, including body temperature exceeding 38°C or falling below 36°C, leukocytosis and increased heart and respiratory rates. (2) Clinical imaging modalities such as computed tomography (CT) or magnetic resonance imaging (MRI) may reveal diffuse cerebral edema, dural thickening and enhancement, or ventricular system dilation. Advanced imaging techniques might also demonstrate characteristic ring-enhancing space-occupying lesions and low-density purulent cavities. (3) Blood Test: The white blood cell count in the complete blood count exceeded 10×10^9/L, or the proportion of neutrophils was greater than 80%. (4) Lumbar Puncture and CSF Analysis: ① Lumbar puncture revealed an elevated pressure greater than 200 mm H_2O. ② The CSF appeared turbid, yellow, or characteristically purulent; if compartmentalized, it could be clear and transparent. ③ The total white blood cell count in the CSF was greater than 100 to 1000×10^6/L, with the proportion of polymorphonuclear leukocytes exceeding 70%. ④ The CSF analysis revealed a reduced glucose concentration, defined as glucose levels below 2.6 mmol/L, with a CSF-to-serum glucose ratio of less than 0.66. Additionally, the CSF protein concentration exceeded 0.45 g/L. ⑤ Furthermore, the CSF culture yielded a positive result.

The Workflow of mNGS

In our laboratory, we developed a sequencing platform utilizing a commercial solution provided by Vision Medicals (http://www.visionmedicals.com/). The extraction processes for DNA and RNA were conducted independently. DNA extraction was performed using a column-based method, with the extraction and purification reagents supplied by Vision Medicals (reagent product number: VM002-50). Conversely, RNA extraction was carried out using a magnetic bead-based method. RNA extraction and purification reagents were procured from Vision Medicals Company (product number: VM006-50). The DNA extraction process did not incorporate an enrichment step; however, RNA underwent enrichment post-extraction. Library construction was performed manually, and DNA fragmentation was achieved using a transposase-based method. The reagents utilized in the library construction process included the Genomic DNA Fragmentation Kit (VM008-50) and the Pathogen DNA Library Construction Special Kit (ILM) RS and VMRS0010-50D. The PCR protocol was as follows: an initial denaturation at 72°C for 3 minutes, followed by 98°C for 30 seconds; 17 cycles of 98°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; a final extension at 72°C for 5 minutes; and a hold at 4°C. It is essential that the reaction be conducted under a hot cover. The bioinformatics analysis procedure commenced with the acquisition of sequencing FASTQ files. Initially, low-quality sequencing data were filtered utilizing the Fastp software. Subsequently, the filtered data were aligned to the human genome employing the BWA software, thereby generating a non-human sequencing dataset. This non-human sequence dataset was then aligned to a pathogen reference database to facilitate species identification and annotation. The bioinformatics analysis workflow encompassed several critical steps: the removal of sequencing adapters, the exclusion of low-quality sequences, the elimination of low-complexity sequences, the filtering of short sequences, the identification and removal of duplicate sequences, the exclusion of human sequences, and the alignment of microbial sequences. The human genome reference database utilized included Human GRCh38/hg38, YH2.0, and the NT library of human sequences. Our laboratory's comprehensive databases encompassed 18,562 microorganisms, comprising 9838 bacteria, 6761 viruses, 1551 fungi, 305 parasites, and 107 mycoplasma and chlamydia species.

Traditional Pathogenic Diagnostic Techniques of CSF

Bacteria and fungi were isolated and identified utilizing cultivation techniques. The identification process for Cryptococcus neoformans involved both cultivation methods and the detection of the Cryptococcus capsule antigen in CSF samples. For Mycobacterium tuberculosis, identification methods encompassed culture, GeneXpert MTB/RIF assay, and smear staining. The cultivation of CSF samples was meticulously conducted following the laboratory's standardized operating procedures. Strain identification was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, AUTOFMS 1000, ANTU, China). Nucleated cell counts in peripheral blood were determined using a fully automated blood analyzer (BC-6800Plus, Mindray, China). The quantification of protein levels in CSF was conducted utilizing a fully automated biochemical analyzer (Cobas 8000 c701, Roche Diagnostics, Germany). In contrast, the cell count in CSF samples was performed manually, with cells being observed and enumerated using microscopic examination.

Statistical Analysis

The diagnostic efficacy of mNGS and conventional pathogenic diagnostic methods for CSF was assessed by calculating sensitivity, specificity, PPV, NPV, and the Youden index. Additionally, ROC curves for the different detection methods were constructed, and the AUC was determined for each method. Statistical analyses were conducted utilizing SPSS software version 22.0 (IBM, Armonk, NY, USA) and GraphPad Prism version 9.5 (GraphPad Software, San Diego, CA, USA). A p-value of less than 0.05 was deemed to indicate statistical significance.

Results

Patient Characteristics

Between November 2021 and October 2022, a total of 68 patients were enrolled in the study, comprising 44 individuals clinically diagnosed with CNSI and 24 non-infected individuals serving as the control group. Analysis of age distribution revealed a significantly higher proportion of patients over 60 years old in the control group compared to the infection group. No significant differences were observed between the two groups concerning gender, underlying medical conditions, history of smoking and alcohol consumption, or prior use of antibiotics before sample collection (see Table 1).

Table 1 Demographic and Clinical Characteristics of the 68 Patients

Characteristic	Value		P
	Infection Group (n=44)	Control Group (n=24)	
Age			
Mean — yr	42	50	
Distribution — no. (%)			
0–12 yr	2 (4.5)	0 (0)	
13–18 yr	4 (9.1)	1 (4.2)	>0.05
19–25 yr	1 (2.3)	3 (12.5)	>0.05
26–40 yr	17 (36.6)	4 (16.7)	>0.05
41–60 yr	18 (40.9)	5 (11.4)	>0.05
>60 yr	2 (4.5)	11 (25.0)	0.01<P<0.05

(*Continued*)

Table 1 (Continued).

Characteristic	Value		P
	Infection Group (n=44)	Control Group (n=24)	
Male sex — no. (%)	32 (72.7)	13 (54.2)	>0.05
Underlying disease			
Diabetes — no. (%)	5 (11.4)	1 (4.2)	>0.05
Hypertension — no. (%)	4 (9.1)	4 (16.7)	>0.05
Chronic nephrotic syndrome — no. (%)	1 (2.3)	0 (0)	
Hyperthyroidism — no. (%)	2 (4.5)	0 (0)	
Rheumatoid arthritis — no. (%)	2 (4.5)	0 (0)	
Congenital muscular dystrophy — no. (%)	1 (2.3)	0 (0)	
Long term smoking and/or drinking — no. (%)	2 (4.5)	0 (0)	
Use antibiotics before testing — no. (%)	44 (100)	24 (100)	
Cases of deaths — no. (%)	1 (2.3)	1 (4.2)	>0.05

Diagnostic Performance Evaluation of Different Detection Methods

mNGS demonstrated the highest sensitivity among single detection methods, with a sensitivity rate of 67.6% (95% confidence interval [CI] 50.85–80.87%). However, its specificity was limited to 45.8% (95% CI 27.89–64.92%). In contrast, the cultivation method exhibited the highest specificity at 100%, though its sensitivity was considerably lower at 10.6% (95% CI 4.63–22.6%). Notably, the combined application of mNGS and traditional techniques yielded a diagnostic sensitivity of 91.3% (95% CI 79.67–96.56%) and a specificity of 18.2% (95% CI 7.31–38.51%) (refer to Table 2). The areas under the ROC curve for culture, peripheral blood nucleated cell count, mNGS, CSF nucleated cell

Table 2 Diagnostic Efficiency of Different Methodologies

Diagnostic Method	Sensitivity (%) 95% CI	Specificity (%) 95% CI	PPV (%) 95% CI	NPV (%) 95% CI	Youden Index (%)
Total enrolled patients (n=68)					
Culture	10.6 (4.63–22.6)	100 (84.54–100)	19.2 (8.51–37.88)	0 (0–8.38)	10.6
mNGS	67.6 (50.85–80.87)	45.8 (27.89–64.92)	67.6 (50.85–80.87)	38.2 (23.9–54.96)	13.4
WBC (CSF)	66.0 (51.67–77.83)	61.9 (40.87–79.25)	70.5 (55.78–81.84)	33.3 (17.97–53.29)	27.9
WBC (blood)	34.0 (22.17–48.33)	57.1 (36.54–75.53)	57.1 (39.07–73.49)	22.5 (12.32–37.5)	−8.9
TP (CSF)	63.8 (49.54–76.03)	57.1 (36.54–75.53)	71.4 (56.43–82.83)	34.6 (19.42–53.78)	20.9
The combination of the above methods	91.3 (79.67–96.56)	18.2 (7.31–38.51)	91.3 (79.67–96.56)	81.8 (61.49–92.69)	9.5
Number of nuclear cells in CSF<8 (n=28)					
mNGS	50.0 (26.8–73.2)	53.3 (30.11–75.19)	46.7 (24.81–69.89)	53.8 (29.15–76.8)	3.3
Number of nuclear cells in CSF≥8 (n=40)					
mNGS	51.6 (34.84–68.03)	33.3 (12.06–64.58)	84.2 (62.43–94.48)	28.6 (13.81–49.95)	−15.1

Abbreviations: PPV, Positive predictive value; NPV, Negative predictive value; WBC, white blood cell; CSF, cerebrospinal fluid; TP, Total protein; mNGS, metagenomic next-generation sequencing.

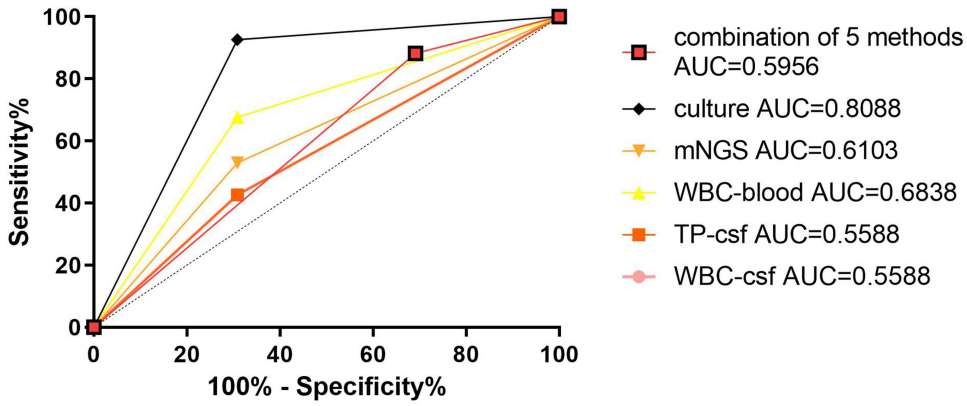

Figure 1 Presents the Receiver Operating Characteristic (ROC) curves illustrating the diagnostic efficacy of different methodologies for detecting central nervous system infections. Here, "WBC blood" denotes the nucleated cell count in peripheral blood, "WBC-CSF" refers to the nucleated cell count in cerebrospinal fluid, and "TP-CSF" indicates the protein content within the cerebrospinal fluid.

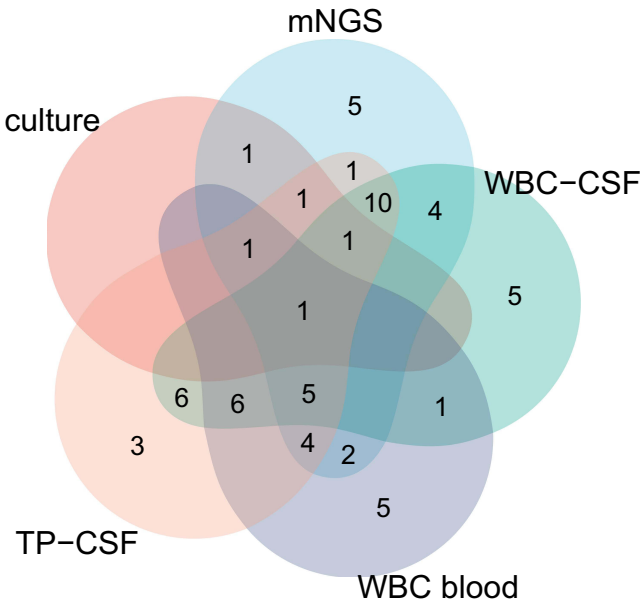

Figure 2 Presents a Venn diagram comparing the outcomes of mNGS with those of conventional diagnostic tests. In this context, "WBC blood" denotes the count of nucleated cells in peripheral blood, "WBC-CSF" refers to the count of nucleated cells in cerebrospinal fluid, and "TP-CSF" indicates the protein content in cerebrospinal fluid.

count, and CSF protein content were 0.8088, 0.6038, 0.6103, 0.5588, and 0.5588, respectively (see Figure 1). Figure 2 illustrates the distribution of consistency among samples for mNGS, peripheral blood nucleated cell count, CSF nucleated cell count, positive CSF protein content, and culture results. Figures S1 and S2 depict the distribution of pathogen types identified by mNGS and culture methods, respectively.

Discussion

Our research indicates that existing laboratory testing methods, encompassing both traditional techniques and mNGS, remain suboptimal for the diagnosis of CNSI. The laboratory diagnosis of CNSI continues to encounter substantial challenges. While mNGS has demonstrated certain advantages, its sensitivity and specificity require enhancement. Furthermore, the detection process of mNGS necessitates ongoing optimization.

mNGS has garnered significant attention as a novel diagnostic approach. A multicenter study conducted by M. R. Wilson on the application of mNGS in diagnosing encephalitis and meningitis demonstrated that conventional diagnostic methods

often fall short in identifying common pathogens associated with CNSI.[11] The study further indicated that mNGS has the potential to address the limitations of traditional techniques in diagnosing certain patients.[11] Furthermore, a study conducted by Zhang Yi demonstrated that mNGS exhibited commendable diagnostic performance in CNSI and surpassed traditional culture methods in overall detection rates.[13] The research also revealed that the detection rate of mNGS was significantly elevated in patients presenting with cerebrovascular disease who had CSF white blood cell counts exceeding 300×10^6/L, CSF protein levels greater than 500 mg/L, or a glucose ratio of ≤ 0.3.[13]

In actual clinical settings, discrepancies between the outcomes of mNGS and conventional methodologies in diagnosing CNSI have become more prevalent.[14] However, our study found that mNGS did not exhibit significant advantages over traditional techniques, such as CSF nuclear cell counting and CSF protein analysis. By integrating traditional technology with mNGS technology, the diagnostic sensitivity was enhanced to 91.3%; however, the specificity remained low at 18.2%. Several factors may contribute to the suboptimal mNGS detection outcomes: (1) CSF samples were collected via lumbar puncture rather than directly from the lesion site, potentially resulting in a lower pathogen load compared to samples obtained directly from the lesion. The efficacy of pathogen detection via mNGS was notably influenced by the elevated proportion of host DNA present in the samples. Furthermore, the sample volume in each tube significantly impacted the performance of various detection methods, including GeneXpert MTB/RIF, culture, and mNGS, all of which have stringent requirements regarding sample volume. The quantity of nucleic acids extracted and the volume of data generated during bioinformatics analysis both significantly influence the detection efficacy of mNGS.

As a non-targeted pathogen detection technology, mNGS demonstrates substantial advantages in identifying rare pathogens. Additionally, it is capable of detecting co-infections involving bacteria, fungi, viruses, and parasites, making it particularly suitable for patients with compromised immune systems and those in critical condition.[15] However, concurrently, it exhibited several evident limitations. For instance, in sterile body fluid samples such as CSF, the nucleic acid fragments of pathogens were notably short, posing challenges for mNGS technology in capturing these brief nucleic acid sequences.[16] A study conducted by Han Dongsheng assessing the diagnostic efficacy of mNGS in bloodstream infections revealed that the clinical correlation of negative mNGS test results was merely 32.4%.[17] The utilization of negative results from mNGS to exclude infection was associated with a substantial risk of missed detection. The application of mNGS in diagnosing bloodstream infections continues to encounter significant challenges. Interpreting mNGS results remains particularly complex, especially for pathogens with low sequence abundance. Ensuring the accuracy of mNGS results presents a highly thought-provoking issue. The verification of detection results from each mNGS assay, incorporating methods such as Sanger sequencing and conventional pathogen diagnostic techniques, represents an optimal scenario that is largely impractical in clinical settings. Evaluating the clinical relevance of mNGS findings necessitates a holistic assessment, encompassing the patient's clinical presentation, medical history, imaging studies, and antibiotic usage.[17,18] In comparison to traditional culture methods, mNGS exhibited a higher rate of false positives.[8] However, there were instances where culture methods yielded positive results that mNGS failed to detect.[9] Notably, mNGS demonstrated a significantly faster detection speed for common pathogens than conventional techniques like culture.[19] In comparison to conventional pathogenic diagnostic methods, mNGS offers substantial advantages in detecting fungi, *M. tuberculosis*, viruses, anaerobic bacteria, and atypical pathogens.[20,21] Consequently, in the context of diagnosing infectious diseases, mNGS and traditional diagnostic techniques serve as complementary approaches.

In this study examining CNSI, the clinical final judgment served as the benchmark for assessing both mNGS and conventional pathogenic diagnostic methods. Our findings indicate that mNGS, alongside traditional techniques such as culture, blood nucleated cell detection, CSF nucleated cell detection, and CSF protein quantification, whether used independently or in combination, did not adequately fulfill the clinical diagnostic criteria. Consequently, the accurate pathogenic diagnosis of CNSI continues to encounter substantial challenges.

This study was subject to several limitations. Firstly, the mNGS detection results had not been corroborated through diverse methodologies, such as Sanger sequencing. The interpretation of mNGS results relied on the CCI. Secondly, the retrospective design of the study led to suboptimal patient enrollment compared to prospective studies, consequently diminishing the diagnostic efficacy characteristic of evidence-based medicine relative to prospective research designs.

Conclusions

In actual clinical practice, mNGS has not demonstrated satisfactory diagnostic efficiency for CNSI. Similarly, conventional pathogenic diagnostic methods, including culture, along with auxiliary diagnostic techniques such as CSF nucleated cell count, CSF protein content, and peripheral blood nucleated cell count, have also failed to achieve satisfactory diagnostic efficiency. Consequently, the laboratory diagnosis of CNSI continues to encounter significant challenges.

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Disclosure

The authors report no conflicts of interest in this work.

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