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ORIGINAL RESEARCH

Exploring the Spectrum of Microbiota in Central Nervous System Infections Through Metagenomic Next-Generation Sequencing

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Purpose: This study leveraged CSF metagenomic next-generation sequencing (mNGS) to bridge this knowledge gap and elucidate the microbiota spectrum of CNS infections.

Patients and Methods: We retrospectively analyzed CSF mNGS reports and clinical data from 264 patients with suspected CNS infections, who were enrolled from September 2019 to November 2023.

Results: According to diagnostic criteria, 145 patients were diagnosed with CNS infections, including bacterial (27 cases, 18.6%), *Mycobacterium tuberculosis* (30, 20.7%), fungal (23, 15.9%), and viral (65, 44.8%) infections. The mNGS positive detection rate was 46.2% (67/145), with significant differences among groups ($p < 0.001$). A total of 22 pathogens were identified, most commonly *Cryptococcus neoformans* (16, 23.9%), *Mycobacterium tuberculosis* (10, 14.9%), and Epstein-Barr virus (9, 13.4%). The most frequent background microorganisms detected by mNGS were *Cutibacterium acnes* (58.6%), *Moraxella osloensis* (29.0%), and *Malassezia restricta* (26.2%).

Conclusion: High-throughput sequencing using mNGS revealed the microbial compositions in CSF samples from patients with CNS infections. This approach may enhance our understanding of pathogens and assist clinicians in making effective therapeutic decisions.

Keywords: central nervous system infection, microbiota, metagenomic next-generation sequencing, cerebrospinal fluid

Introduction

Central nervous system (CNS) infections, such as meningitis, encephalitis, and brain abscesses, are severe diseases with high disability and mortality rates. These infections primarily include viral encephalitis and meningitis, tuberculous meningitis (TBM), bacterial meningitis, and fungal meningitis. Patients often present with symptoms such as fever, headache, vomiting, neck stiffness, altered consciousness, and convulsions.[1] The overlapping clinical phenotypes and cerebrospinal fluid (CSF) findings—such as increased intracranial pressure, elevated WBC count, and abnormal glucose and protein levels—make it challenging to diagnose the specific etiology of CNS infections using traditional methods. Moreover, previous epidemiological studies have shown that approximately 50% of patients with CNS infections do not receive a definitive diagnosis.[2–5] Research on the microbiota of intracranial infections is limited, with existing reports mainly focusing on specific pathogens. Conventional diagnostic techniques often reveal only 'the tip of the metagenomic iceberg", leading to inappropriate antibiotic therapies. Therefore, it is crucial to explore the full microbial spectrum present in intracranial infections to improve diagnostic accuracy and treatment efficacy.

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Metagenomic next-generation sequencing (mNGS) offers a hypothesis-free approach to detect a wide array of pathogens, including bacteria, fungi, viruses, and parasites. This method can identify pathogens that may not be initially considered by neurologists due to their rarity, lack of association with known clinical manifestations, or recent discovery as new organisms.[6–8] Additionally, mNGS is less impacted by prior antibiotic exposure.[9,10] However, most existing data on the epidemiology of CNS infections focus on specific pathogen types.[11,12] There is currently a lack of research on the application of mNGS for analyzing the microbiota involved in CNS infections. To address this gap, we conducted a retrospective study to gain a more comprehensive understanding of the etiology of CNS infections using mNGS.

Materials and Methods

Subjects

For this retrospective study, we enrolled 264 consecutive cases with suspected CNS infections from the First Affiliated Hospital of Fujian Medical University between September 2019 and November 2023. We based the clinical criteria for CNS infections on the diagnostic guidelines for meningitis, encephalitis, meningoencephalitis, and meningomyelitis, as reported in previous studies. These criteria included symptoms such as fever, headache, seizures, altered consciousness, signs of meningeal irritation, and new onset of focal neurological findings.[13,14]

Two clinicians independently assessed all clinical information for the final diagnosis. We excluded cases with autoimmune neurological diseases, epilepsy, cerebrovascular disease, intracranial tumors, other non-infectious neurological diseases, CNS infections with special pathogens (such as Creutzfeldt-Jakob disease or neurosyphilis), non-neurological diseases, and diseases with unknown diagnoses. Out of the 264 patients, we selected 145 eligible individuals with confirmed CNS infections for further analysis. We divided these patients into four groups: bacterial infections of the CNS (n = 27), tuberculosis infections of the CNS (n = 30), fungal infections of the CNS (n = 23), and viral infections of the CNS (n = 65). Figure 1 illustrates the study enrollment flow chart. We obtained informed consent from all enrolled patients, and the protocol received approval from the Ethics Committee of the First Affiliated Hospital of Fujian Medical University.

CSF Sampling and Routine Testing

We collected cerebrospinal fluid (CSF) via lumbar puncture following standard procedures and immediately placed it in sterile container. We then aliquoted the CSF samples into 1.5 mL sterile Eppendorf tubes and stored them at 4 °C for subsequent assays. Routine CSF testing included cytology, biochemistry, bacterial and fungal smears and cultures, India Ink preparation, acid-fast staining, and cryptococcal antigen (CrAg) testing. These tests were performed at the Laboratory Medicine Center of the First Affiliated Hospital of Fujian Medical University. We strictly adhered to sterile protocols during specimen collection and transportation. We transported a total of 264 CSF samples to the Beijing Genomics

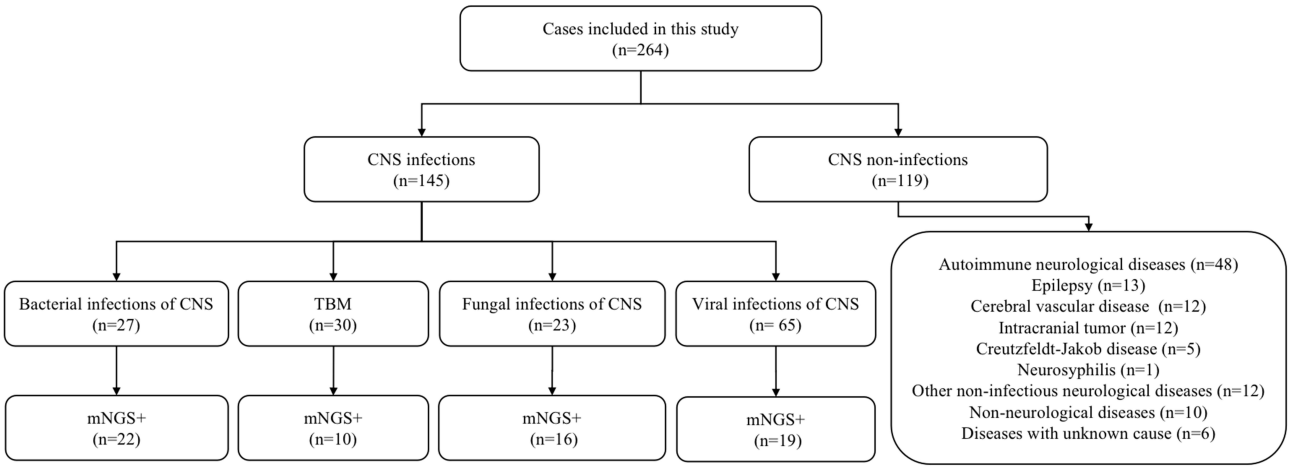

Figure 1 Flowchart of study enrollment.

Institute (BGI, Beijing, China) at 4 °C for mNGS analysis. Throughout the mNGS process, the researchers were blinded to all clinical information, including laboratory results and final clinical diagnoses.

Conventional Microbiological Techniques

For bacterial and fungal culture, samples were inoculated onto blood, chocolate, and Sabouraud agar plates. They were incubated under appropriate atmospheric conditions (eg, 37°C, 5% CO_2). Isolated colonies were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or standard biochemical tests. For cryptococcal antigen (CrAg) testing, CSF samples were analyzed using the Latex Cryptococcus Antigen Detection System (IMMY Norman, USA) according to manufacturer protocols. Each sample was independently analyzed by two trained technicians with validated concordance. Results were reported semi-quantitatively as titre levels.

Metagenomics Next-Generation Sequencing Detection

We added 600 μL of CSF samples to 7.2 μL of Lyticase (RT410-TA, TIANGEN BIOTECH, Beijing, China) to facilitate enzyme wall breaking. Subsequently, we added 250 μL of 0.5 mm glass beads for physical wall disruption. After thorough mixing and shaking, we extracted 300 μL of the samples using the TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH, Beijing, China).[7] We processed the extracted nucleic acid through enzyme digestion, terminal repair, splicing, and PCR reaction to construct the library. We used the Agilent 2100 Bioanalyzer to ensure the library fragments were approximately 300 bp in size. Using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific Inc)., we measured the DNA library concentration controlled and pooled the constructed library samples to ensure uniform quality based on concentration. After pooling, we cycled the library mixture to form a unit chain ring structure and generated DNB nanospheres through rolling circle amplification (RCA). We then loaded the prepared DNB nanospheres into the sequencing chip and performed sequencing using the BGISEQ-50/MGISEQ-2000 platforms.[8] We first removed low-quality sequences from the sequencing data after unloading the sequencing data to obtain high-quality data. We used the BWA tool (bio-bwa.sourceforge.net/) to eliminate reference genomic sequences from the high-quality data.[9] After excluding low-complexity reads, we compared the remaining data against the BTU PMDB pathogen database, which includes 6350 bacteria, 1064 fungi, 4945 viruses, and 234 parasites. This comparison allowed us to determine the sequence counts and identify potential pathogens based on these counts and other clinical tests. We considered microorganisms to be credible if they met the following criteria: For bacteria (excluding *Mycobacterium tuberculosis* (MTB)), fungi, DNA viruses, and parasites, the microbe had a minimum of three non-redundant mapped reads per 10 million raw sequence reads. For RNA viruses and MTB, due to the difficulty of detection, we reported the samples as positive when at least one specific, high-quality sequence was identified.

Statistics Analysis

We expressed continuous variables with normal distributions as mean ± SD, while we presented continuous variables with non-normal distributions as medians and interquartile ranges (IQRs). We expressed categorical variables as counts (no.) and percentages (%). We evaluated differences in continuous variables using analysis of variance (ANOVA) for Gaussian distributions and the Mann–Whitney or Kruskal–Wallis tests for non-Gaussian distributions. We performed statistical analyses using GraphPad Prism software (version 8.0) and SPSS 26.0 software (IBM Corp, Armonk, NY, United States). We considered a P value < 0.05 statistically significant, without adjusting for multiple testing.

Results

Sample and Patient Characteristics

Between September 2019 and November 2023, we collected CSF samples from 264 patients with suspected CNS infections for mNGS detection (Figure 1). We excluded 119 samples that were not consistent with CNS infections, including 48 cases with autoimmune neurological diseases, 13 cases with epilepsy, 12 cases with cerebrovascular disease, 12 cases of intracranial tumors, 5 cases of Creutzfeldt-Jakob disease, 1 case of neurosyphilis, 12 cases of other non-infectious neurological diseases, 10 cases of non-neurological diseases, and 6 cases with unknown causes (Figure 1). We

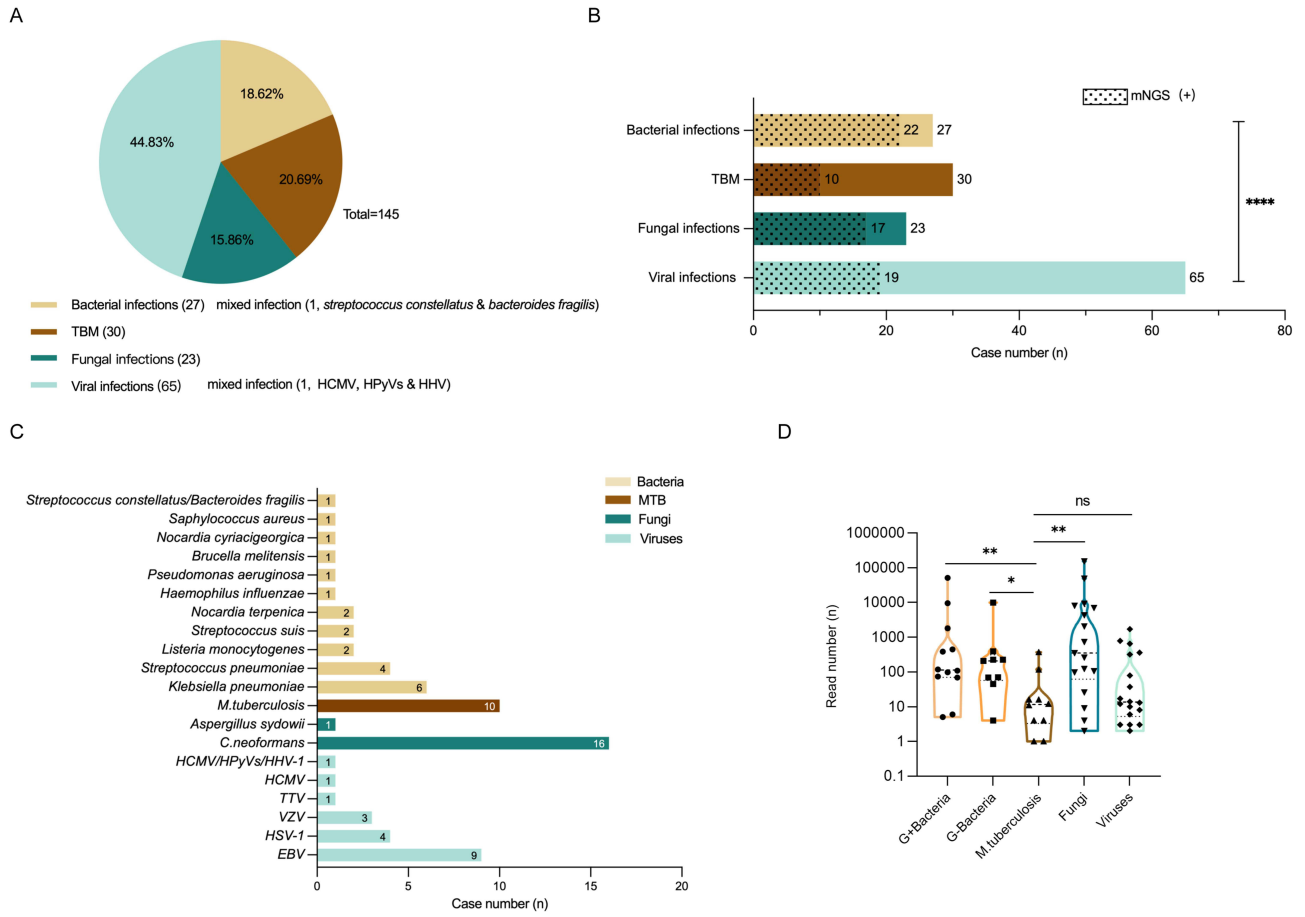

Figure 2 Results of the metagenomic next-generation sequencing. (**A**) Distribution of different types of microbial infections. (**B**) Positivity rates of mNGS in different infectious groups. (**C**) Frequencies for 22 different species of pathogens detected in different infectious groups. (**D**) Read numbers of mNGS detected in different infectious groups.
Note: *p<0.05; **p<0.01; ****p<0.0001.

finally enrolled 145 samples confirmed as CNS infections, comprising 27 cases of bacterial infections (18.62%), 30 cases of TBM (20.69%), 23 cases of fungal infections (15.86%), and 65 cases of viral infections (44.83%) (Figure 2A).

Table 1 presents the demographic characteristics of the patients with CNS infections in this study. Of the patients, 70.34% were male, with an average age of 51 years, ranging from 14 to 88 years. The primary neurological symptoms observed were fever (105/145, 72.41%), headache (96/145, 66.21%), vomiting (55/145, 37.93%), altered consciousness (60/145, 41.38%), neck stiffness (89/145, 61.38%), and convulsions (34/145, 23.45%). Fever was the most prevalent symptom among the subjects in this study. Statistically significant differences were noted in the occurrence of headache ($p = 0.007$), neck stiffness ($p = 0.018$), and convulsions ($p = 0.039$) among the groups. Furthermore, there were significant differences in intracranial pressure increases among the groups, with the bacterial infection group exhibiting the most substantial increase.

Laboratory tests of CSF showed significant variations across the groups in white blood cell count, mononuclear cell percentage, protein, and glucose levels ($p < 0.01$). Specifically, the bacterial infection group had notably higher white blood cell and protein counts compared to the other groups, while glucose levels were lower. Additionally, the bacterial infection group had a significantly lower mononuclear cell count compared to the other groups. Previous medical histories included diabetes mellitus, immunosuppression, cancer, rheumatic disease, and the use of glucocorticoids or immunosuppressants. However, the differences in these conditions among the groups were not statistically significant.

Table 1 Clinical Characteristics and CSF Laboratory Examinations of the Enrolled Cases

	CNS Infections (n=145)	Bacterial Infections of CNS (n=27)	TBM (n=30)	Fungal Infections of CNS (n=23)	Viral Infections of CNS (n=65)	p Value
Gender, (%)						0.641
Male	102 (70.34)	18 (66.67)	19 (63.33)	15 (65.22)	50 (76.92)	
Female	43 (29.66)	9 (33.33)	11 (36.67)	8 (34.78)	15 (23.08)	
Age (range)	51 (14–88)	54 (25–81)	51 (21–73)	54 (21–77)	48 (14–88)	0.381
Clinical Manifestation, n (%)						
Fever	105 (72.41)	22 (81.48)	22 (73.33)	15 (65.22)	46 (70.77)	0.771
Headache	96 (66.21)	14 (51.85)	24 (80.00)	21 (91.30)	37 (56.92)	0.007
Vomiting	55 (37.93)	16 (59.26)	12 (40.00)	9 (39.13)	18 (27.69)	0.085
Disturbance of consciousness	60 (41.38)	15 (55.56)	11 (36.67)	4 (17.39)	30 (46.15)	0.073
Neck stiffness	89 (61.38)	21 (77.78)	22 (73.33)	16 (69.57)	30 (46.15)	0.018
Convulsions	34 (23.45)	5 (18.52)	4 (13.33)	2 (8.70)	23 (35.38)	0.039
CSF laboratory test, median (range)						
Intracranial pressure (mmH_2O)	160 (30–330)	200 (60–330)	160 (50–330)	175 (60–330)	150 (30–330)	0.018
CSF WBC ($*10^6$/L)	87 (1–433373)	472 (1–433373)	116 (37–1061)	102 (18–325)	19 (1–398)	< 0.001
Monocyte ratio (%)	86.75 (6.8–100)	43.9 (6.8–100)	86.6 (34.7–100)	86.8 (59.5–100)	92.3 (7.1–100)	< 0.001
CSF Protein (g/L)	0.81 (0.1–24.49)	1.535 (0.1–24.49)	1.07 (0.52–3.63)	0.8 (0.16–3.91)	0.58 (0.22–2.23)	< 0.001
CSF glucose (mmol/L)	2.75 (0.05–11.43)	2.1 (0.05–11.43)	2.2 (0.9–3.1)	2.18 (0.49–8.22)	3.53 (1.91–8.57)	< 0.001
Empirical anti-infective drugs, n (%)	77 (53.10)	16 (59.26)	14 (46.67)	10 (43.48)	37 (56.92)	< 0.001
Diabetes, n (%)	19 (13.10)	6 (22.22)	3 (10.00)	3 (13.04)	7 (10.77)	0.674
Immunosuppressive state, n (%)						
Malignant tumor	7 (4.83)	4 (14.81)	0 (0.00)	0 (0.00)	2 (3.08)	0.073
Rheumatic disease	11 (7.59)	3 (11.11)	1 (3.33)	4 (17.39)	3 (4.62)	0.072
Glucocorticoids	28 (19.31)	4 (14.81)	9 (30.00)	5 (21.74)	11 (16.92)	0.734
Immunosuppressor	9 (6.21)	3 (11.11)	1 (3.33)	3 (13.04)	2 (3.08)	0.130

Microbial Spectrum Detected by mNGS in CNS Infections

The overall positive rate of mNGS for detecting CNS infections in the enrolled patients was 46.21% (67/145). The detection rates of pathogens by mNGS varied significantly among infection groups ($p < 0.001$). The bacterial infection group had the highest positive rate at 81.48% (22/27), while the viral infection group and the tuberculosis infection group had lower detection rates at 29.23% (19/65) and 33.33% (10/30), respectively. The fungal infection group had a detection rate of 69.57% (17/23) (Figure 2B).

mNGS identified a total of 22 different pathogen species in CNS infections (Figure 2C). In the bacterial infection group, the primary pathogens were *Klebsiella pneumoniae* (6 cases), *Streptococcus pneumoniae* (4 cases), *Nocardia* (3 cases), *Listeria monocytogenes* (2 cases), and *Streptococcus suis* (2 cases). Other pathogens included *Haemophilus influenzae*, *Brucella melitensis*, and *Pseudomonas aeruginosa*, each identified in one case. Additionally, one case involved a mixed infection with *Streptococcus constellatus* and *Bacteroides fragilis*. In the TBM group, MTB was detected in 10 cases.

In the fungal infection group, mNGS predominantly identified *Cryptococcus neoformans* (*C. neoformans*) (69.57%, 16/23 cases), with only one case of *Aspergillus* detected. In the viral infection group, the main pathogens included Epstein-Barr virus (EBV) (9 cases), varicella-zoster virus (VZV) (4 cases), herpes simplex virus type 1 (HSV-1) (3 cases), human cytomegalovirus (HCMV) (1 case), Torque teno virus (TTV) (1 case), and a mixed infection involving HCMV, *Human herpesvirus 1* (HHV-1), and *human polyomavirus* (HPyVs) (1 case). Notably, EBV was also detected in 10 cases in the non-infection group (Figure 3A).

Based on mNGS results, we categorized the pathogens in the bacterial group into Gram-positive bacteria and Gram-negative bacteria. We compared the read numbers for the TBM group with those of other infection groups (Figure 2D and Table 2). The read numbers for MTB (11.5, range 1–368) were significantly lower than those for Gram-positive bacteria

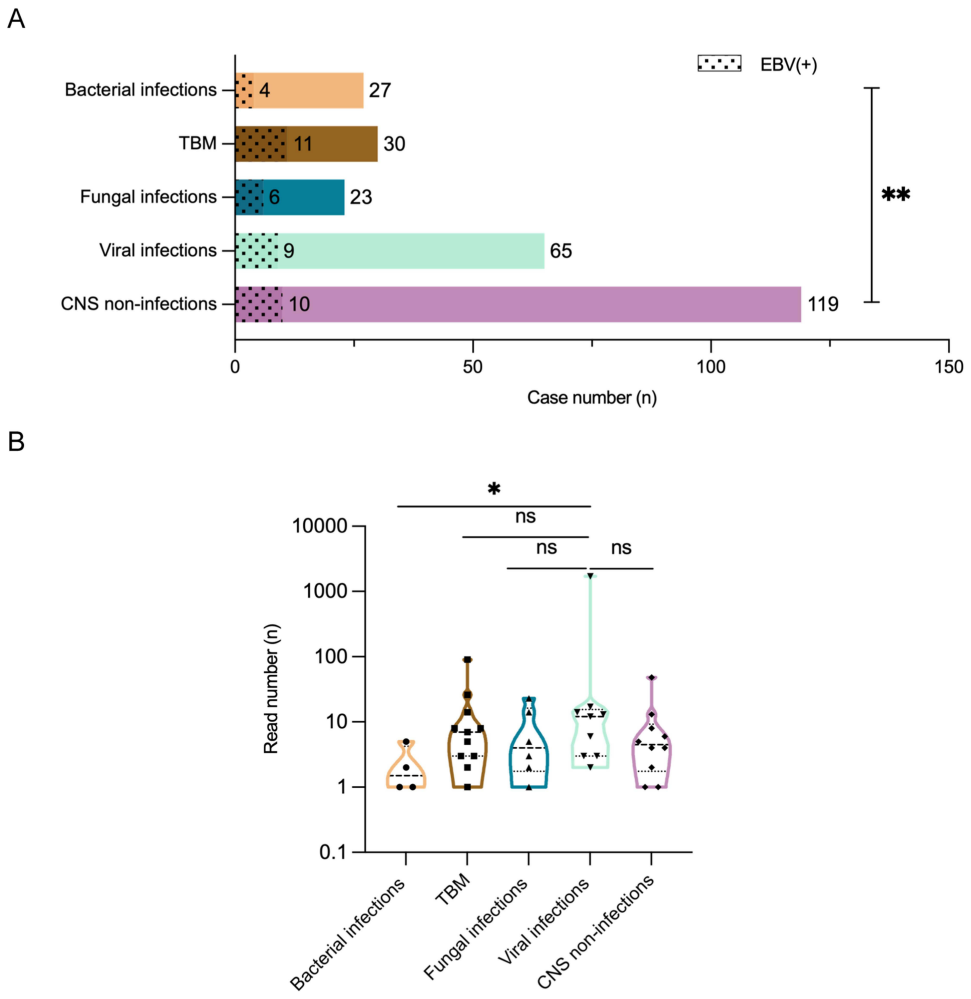

Figure 3 EBV analysis. (**A**) Positivity rates of EBV in different infectious groups detected by mNGS. (**B**) Read numbers of EBV detected in different infectious groups.
Note: *p<0.05; **p<0.01.

(113.5, range 5–51,340), Gram-negative bacteria (208.0, range 4–9919), and fungal groups (350.0, range 2–152,863) (Figure 2D). However, there was no significant difference (p = 0.3619) between the TB group and the viral group (13.5, range 2–1701). Additionally, no significant difference (p = 0.3064) was observed in the read numbers for EBV between the viral infections (196.8, range 2–1701) and CNS non-infections (9.2, range 1–48) (Figure 3B and Table S1). Moreover,

Table 2 Reads of Positive mNGS Cases

	Species	Read Number
G+ bacteria	*Streptococcus pneumoniae*	1803
		5
		98
		443
	Nocardia terpenica	386
		69
	Nocardia cyriacigeorgica	6
	Listeria monocytogenes	109
		118

(*Continued*)

Table 2 (Continued).

	Species	Read Number
	Streptococcus suis	51340
		9512
	Streptococcus constellatus	26
	Saphylococcus aureus	74
G- bacteria	*Klebsiella pneumoniae*	9919
		70
		225
		224
		392
		208
	Pseudomonas aeruginosa	69
	Haemophilus influenzae	4
	Bacteroides fragilis	215
	Brucella melitensis	45
Acid-fast	*M. tuberculosis*	4
		12
		16
		1
		117
		16
		1
		11
		4
		368
Fungi	*C. neoformans*	2071
		98
		49,101
		26
		730
		8146
		9329
		6950
		350
		122
		9
		152,863
		2
		4312
		106
		262
	Aspergillus sydowii	4
Viruses	EBV	
		3
		13
		2
		14
		12
		1701
		17
		6

(*Continued*)

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Table 2 (Continued).

	Species	Read Number
	HSV1	781
		358
		79
		651
	VZV	37
		320
		3
	CMV	3
		10
	HHV-1	3
	HPyV	2
	TTV	8

Abbreviations: G+ bacteria, Gram-positive bacteria; G- bacteria, Gram-negative bacteria; *M. tuberculosis, Mycobacterium tuberculosis; C.neoformans, Cryptococcus neoformans*; EBV, Epstein-Barr virus; HSV1, herpes simplex virus type 1; VZV, varicella-zoster virus; CMV, cytomegalovirus; HHV-1, *Human herpesvirus* 1; HPyV, *human polyomaviruse*; TTV, Torque teno virus.

conventional methods detected pathogens at a positive rate of 13.10% (19/145), which was significantly lower than that of mNGS. This included bacterial smears (3/27, 11.11%) and cultures (4/27, 14.81%) in the bacterial group, RT-PCR (2/17, 11.76%) and Xpert MTB/RIF (1/15, 6.67%) in the TBM group, and India Ink (14/20, 70.00%) and CrAg testing (16/18, 88.89%) in *Cryptococcus* infections.

Background Microbiological Analysis of mNGS Assays

Bacteria and fungi were the most frequently detected background microorganisms by mNGS (Table 3). The top 10 microorganisms, listed in descending order of detection rates, were: *Cutibacterium acnes* (85 cases, 58.62%), *Moraxella*

Table 3 The Cases of Background Microorganisms

	Genus	Cases	Species	Cases
G+ bacteria	*Cutibacterium*	88	*Cutibacterium acnes*	85
G+ bacteria	*Staphylococcus*	69	*Staphylococcus epidermidis*	41
			Staphylococcus hominis	38
			Staphylococcus haemolyticus	5
			Corynebacterium aurimucosum	5
G+ bacteria	*Bacillus*	13	*Bacillus coagulans*	1
			Bacillus cereus complex	1
G+ bacteria	*Streptococcus*	6	*Streptococcus pneumoniae*	2
			Streptococcus mitis	1
			Streptococcus oralis	1
			Streptococcus mutans	1
G- bacteria	*Acinetobacter*	49	*Acinetobacter johnsonii*	22
			Acinetobacter junii	19
			Acinetobacter guillouiae	12
			Acinetobacter lwoffii	7
G- bacteria	*Moraxella*	44	*Moraxella osloensis*	42
			Moraxella atlantae	2
G- bacteria	*Alcaligenes*	29	*Alcaligenes faecalis*	28

(*Continued*)

Table 3 (Continued).

	Genus	Cases	Species	Cases
G- bacteria	*Pseudomonas*	16	*Pseudomonas stutzeri*	10
			Pseudomonas oleovorans	3
			Pseudomonas fluorescens	2
			Pseudomonas otitidis	1
			Pseudomonas monteilii	1
			Pseudomonas mosselii	1
G- bacteria	*Xanthomonas*	6	*Xanthomonas fragariae*	3
			Xanthomonas campestris	3
G- bacteria	*Serratia*	5	*Serratia marcescens*	5
G- bacteria	*Chryseobacteriu*	5	*Chryseobacterium haifense*	4
G- bacteria	*Acidovorax*	4	*Acidovorax delafieldii*	4
Fugus	*Malassezia*	58	*Malassezia restricta*	38
			Malassezia globosa	11
Fugus	*Aspergillus*	5	*Aspergillus sydowii*	4
Fugus	*Cladosporium*	5	*Cladosporium sphaerospermum*	5

osloensis (42 cases, 28.97%), *Staphylococcus epidermidis* (41 cases, 28.28%), *Staphylococcus hominis* (38 cases, 26.21%), *Malassezia restricta* (38 cases, 26.21%), *Alcaligenes faecalis* (28 cases, 19.31%), *Acinetobacter johnsonii* (22 cases, 15.17%), *Acinetobacter junii* (19 cases, 13.10%), *Acinetobacter guillouiae* (12 cases, 8.28%), and *Malassezia globosa* (11 cases, 7.59%) (Figure 4A). The most common Gram-positive and Gram-negative bacteria were *Cutibacterium acnes* (58.62%) and *Moraxella osloensis* (28.97%), respectively. The most common fungus was *Malassezia globosa* (26.21%). Interestingly, the background bacteria detected in the non-infection group were largely consistent with those found in the infection group. These included *Cutibacterium acnes* (90 cases, 75.63%), *Alcaligenes faecalis* (52 cases, 43.70%), *Malassezia restricta* (50 cases, 42.02%), *Staphylococcus hominis* (45 cases, 37.82%), *Moraxella osloensis* (38 cases, 31.93%), *Acinetobacter junii* (30 cases, 25.21%), *Staphylococcus epidermidis* (28

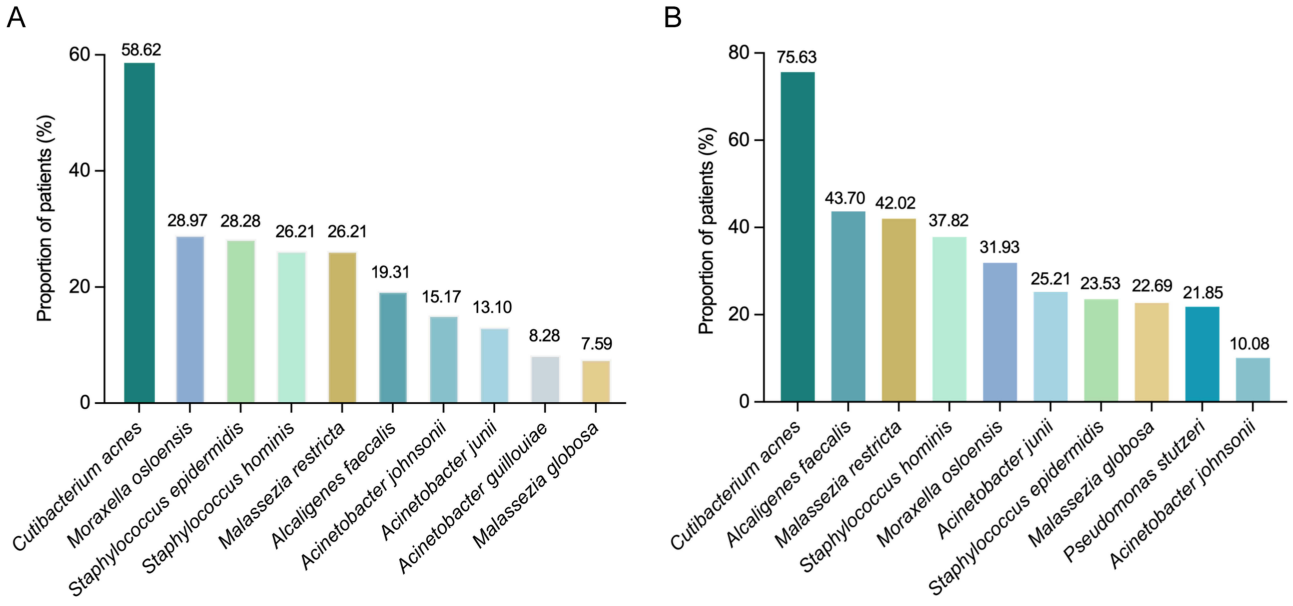

Figure 4 CSF background microbiota analyzed by mNGS. (**A** and **B**) Distribution of the background bacteria detected in the CNS infections group (**A**) and the CNS non-infections group(**B**).

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cases, 23.53%), *Malassezia globosa* (27 cases, 22.69%), *Pseudomonas stutzeri* (26 cases, 21.85%), and *Acinetobacter johnsonii* (12 cases, 10.08%) (Figure 4B).

Discussion

Intracranial infections are common among nervous system disorders. Historically, limitations in detection technology have prevented the identification of pathogens in approximately 50% of patients with these infections.[2–4] This gap has hindered a comprehensive understanding of the range of pathogens associated with intracranial infections. mNGS offers advantages over traditional methods for detecting systemic infectious diseases, such as pulmonary and bloodstream infections. It delivers faster results, can simultaneously detect a broad spectrum of pathogens—including rare ones—and can identify co-infections.[7] In our research, we used mNGS to identify pathogens in CSF, thereby gaining valuable insights into the microbial spectrum of intracranial infections. In our study, for bacterial infections, mNGS identified 22 cases, while culture detected only 3 cases. In TBM, mNGS detected 10 cases, whereas culture yielded no positive results (0 cases). For fungal infections, mNGS identified 17 cases, and culture detected 12 cases. The data highlights the superior sensitivity of mNGS in detecting bacterial and TBM cases compared to culture, while both methods showed relatively closer performance in fungal infection detection (Figure S1).

Bacterial meningitis manifests as a time-critical neurological emergency where delayed pathogen identification correlates with increased mortality and irreversible sequelae. Mortality rates approach 30% in untreated cases and remain substantial (10–30%) despite antibiotic therapy.[15] Failure to diagnose the pathogen promptly and accurately can lead to poor prognosis and high mortality rates. Long-term complications affect up to 50% of survivors of bacterial meningitis.[15] Timely identification, rapid testing, and immediate administration of antibiotics are critical for improving patient outcomes in suspected cases of bacterial meningitis. Notably, between 15% and 68% of patients with bacterial meningitis show negative results on bacterial cultures.[16,17] In our study, the culture positivity rate in patients with bacterial meningitis was only 14.8% (4/27), while mNGS achieved a detection rate of 81.48% (22/27) for bacterial intracranial infections, excluding tuberculous infections. As presented in the Table S2, among the cases, 3 were concordant positive and 5 were concordant negative. Notably, 19 cases were mNGS positive but culture negative. This finding demonstrates the higher sensitivity of mNGS for detecting bacterial pathogens, as it identified infections not detected by culture. Crucially, no cases exhibited mNGS negative but culture positive results, further indicating an extremely low false-negative rate for mNGS in this context, consistent with multiple studies showing mNGS superiority over culture for bacterial detection, especially for polymicrobial infections or difficult-to-culture pathogens.[18] Moreover, mNGS identified a variety of bacterial pathogens, including common ones such as *S. pneumoniae* (4 cases), *L. monocytogenes* (2 cases), and *H. influenzae* (1 case). These findings are consistent with previous studies that used either traditional methods or mNGS for pathogen detection in intracranial infections.[9,16,19] Neisseria meningitis was not detected in our study, which may be due to a reduced incidence of this pathogen following the introduction of the vaccine, a trend also reported in other studies.[20]

Interestingly, our study found that *Klebsiella pneumoniae* was the most commonly detected pathogen in bacterial intracranial infections among patients. Traditionally, *Klebsiella pneumoniae* has been considered rare in community-acquired intracranial infections.[21] However, recent research indicates an increasing prevalence of *Klebsiella pneumoniae* as a causative agent in such cases, particularly in Asia,[21–23] whereas it remains infrequent in Europe. This regional difference may result from the higher prevalence of *Klebsiella pneumoniae* colonization in the intestinal tract of individuals in Asia.[24] Serotypes K1 and K2 of *Klebsiella pneumoniae* have been shown to cause intracranial infections, with previous reports documenting *Klebsiella pneumoniae* invasion syndrome, liver abscesses, sepsis, brain abscesses, and other multi-site lesions.[25,26] In some instances, these serotypes may exclusively cause central nervous system infections.[22] The mortality rate associated with *Klebsiella pneumoniae* infections is significantly high if appropriate antibiotic therapy is not administered promptly.[27]

In addition to detecting common bacteria, our study identified challenging pathogens using mNGS technology. These included *S. suis* (2 cases), *Nocardia* (5 cases), *Brucella* (1 case), and mixed bacterial infections. Traditional clinical methods often struggle to culture *Nocardia* or *Brucella*. For complex intracranial infections involving multiple bacterial pathogens, mNGS technology proves beneficial in preventing diagnostic oversights and enabling timely administration of

appropriate antibiotic therapy. For example, our study demonstrated that mNGS successfully identified a patient with a mixed infection involving *Streptococcus constellatus* and *Bacteroides fragilis*, enabling timely and targeted antibiotic therapy. Notably, two cases of *S. suis* meningitis were rapidly diagnosed within three days via cerebrospinal fluid mNGS, despite remaining undetected by initial conventional methods. Epidemiological observations highlight that *S. suis* meningitis incidence is substantially higher in Southeast Asia compared to Europe, primarily associated with exposure to undercooked pork or contact of open wounds with raw pork products.[28,29] One patient in this study, a pork processor, was presumed infected through hand wounds, while the transmission route for the second case remained undetermined.[30] Following pathogen identification and tailored treatment, both patients showed clinical improvement without developing typical complications such as hearing loss, endocarditis, or intraocular infections. These findings underscore the utility of mNGS in minimizing diagnostic oversight, especially in complex intracranial infections, and facilitating prompt, effective therapeutic interventions.

Our research found that MTB was the most prevalent bacterial pathogen in intracranial infections, accounting for 30 out of 57 cases. This result aligns with findings from other studies on intracranial infections.[9,16] Using mNGS, we detected MTB in 33.33% of cases (10 out of 30). The number of DNA sequences detected was relatively low, with an average of 11.5 reads per sample; most cases (40.0%, 4/10) had ≤4 reads compared to other bacteria and fungi. Previous studies have reported that the sensitivity of mNGS detection ranges from 59% to 84%.[31–33] In contrast, traditional culture methods have shown lower detection rates for MTB, including AFB cultures (0%), conventional cultures (0%), MTB PCR (24.4%), and Xpert MTB/RIF (14.29%).[32,34] MTB is an intracellular pathogen with a cell wall rich in lipids, particularly mycolic acid surrounding the peptidoglycan layer.[35] This lipid barrier complicates DNA extraction, making MTB harder to detect compared to other bacteria.[36] Although mNGS has enhanced the diagnosis of TBM, further improvements are needed, which will require advancements in mNGS technology.

In our study, mNGS identified *Cryptococcus* in 69.57% (16/23) of cases of intracranial fungal infections. This finding may be influenced by the relatively small sample size of our study. Previous research has shown that *Cryptococcus* is the predominant pathogen in intracranial fungal infections, accounting for approximately 70.1% of cases.[37] Other common opportunistic fungi include *Aspergillus*, *Mucor*, and *Candida*. In this study, mNGS also detected one case of *Aspergillus* infection. Risk factors for CNS fungal infections include immunocompromised states, certain medical conditions, and environmental factors.[38] Traditional diagnostic methods often have difficulty identifying these infections; however, mNGS has demonstrated potential for improving diagnostic accuracy.[9,39,40] Although cryptococcal meningitis has declined among HIV patients globally, it still represents 19% of AIDS-related deaths.[38] The incidence of non-HIV cryptococcal meningitis is increasing in high-income countries, likely due to greater use of immunosuppressive therapies and an aging population.[41] The high diagnostic rate for *Cryptococcus* is attributed to the accuracy of the CrAg test, which has a sensitivity of 97.4–100% and is unaffected by antibiotic use, compared to Indian ink staining (63.0%) and culture tests (76.7%).[40,42] However, CrAg testing can produce false positives, whereas mNGS does not. Furthermore, mNGS can differentiate between *Cryptococcus gattii* and *Cryptococcus neoformans* at the species level, which CrAg testing cannot.[43] Thus, mNGS is a promising alternative for detecting fungal DNA and should be considered as a front-line CSF test for fungal meningitis.

In this study, CSF mNGS identified primarily EBV (9 cases), HSV-1 (4 cases), and VZV (3 cases) as pathogenic viruses. EBV emerged as the most common virus in CNS viral infections, which contrasts with findings from other studies.[44] EBV is detectable in 90% of the population and can affect both immunocompromised and immunocompetent individuals.[45,46] Typically, EBV remains latent in B lymphocytes without causing symptoms.[46] Some studies regard detected EBV as background contamination.[47,48] However, primary EBV infection or reactivation of latent EBV can lead to CNS disease in patients with immune deficiencies, as well as in a small number of individuals with normal immune function.[45,46] CNS EBV infections are rare and often lack specific clinical symptoms.[46] To evaluate active EBV replication, CSF can be tested for lytic cycle mRNA.[49] Reactivation of EBV can be monitored by detecting anti-early antigen (EA) antibodies or immunoglobulin A (IgA) anti-viral capsid antigen (VCA) antibodies.[50] The precise number of EBV DNA sequences in CSF required to confirm EBV as the causative agent remains unclear. In HIV-infected patients, the amount of EBV DNA in CSF correlates with the severity of inflammatory brain damage.[51] We observed no significant difference in EBV DNA reads between patients with EBV-related viral infections and those without, which

is consistent with findings from other studies.[52,53] Future evaluations of EBV infections should consider clinical symptoms, investigational treatments, and the detection of EBV antibodies and RNA in both CSF and serum.[52]

Our research identified a low positivity rate and detected limited viral DNA sequences through mNGS, consistent with findings from previous studies on pathogen detection. This observation likely results from the minimal amount of viral DNA present in CSF samples. Existing literature highlights challenges in using metagenomic analysis tools for viral infections with cycle threshold (CT) values ≤28.[54] DNA amplification and fragment length polymorphism (DNA-AFLP) next-generation sequencing (VIDISCA-NGS) offers a potential solution to the problem of low viral load and may be a more effective method for viral metagenomic detection.[44,55] Notably, the viruses identified through mNGS in our study were exclusively DNA viruses, with no RNA viruses detected. Several factors contribute to this outcome: limited submission of RNA tests for detection, the inherent fragility and instability of RNA during storage and transportation leading to potential molecular degradation,[56] and the need for reverse transcription of RNA viruses before deep sequencing, which may result in reduced quantities of detectable DNA fragment.[57]

This study identified similar CSF background microbiota in both the CNS infections group and the non-infections group, including *Cutibacterium acnes*, *Moraxella osloensis*, *Staphylococcus epidermidis*, and *Malassezia restricta* etc. The technique employed is highly sensitive and can detect microorganisms originating from the environment, skin, or reagents.[8] Similar background microbiota had been detected in blood and sputum samples using mNGS. These microorganisms may act as opportunistic pathogens, potentially leading to intracranial infections under specific clinical conditions. For example, *Staphylococcus aureus* and *Staphylococcus hominis* have been associated with central nervous system infections in patients with otitis media, sinusitis, or a history of brain surgery.[58,59] Furthermore, reports suggest that *Candida* and *Acinetobacter* may contribute to iatrogenic intracranial infections.[60,61] Immunocompromised patients are particularly vulnerable to infectious diseases caused by colonized microorganisms and opportunistic pathogens. Therefore, clinicians should exercise caution when interpreting mNGS results, taking into account the presence of background microorganisms. The decision to use anti-infective drugs for background bacteria should be guided by the patient's medical history, imaging results, and laboratory tests of CSF.

The integration of mNGS holds transformative potential for global diagnostic paradigms, albeit with distinct implementation pathways across healthcare settings. In developed countries, mNGS is poised to augment critical care algorithms (eg, sepsis, encephalitis) by delivering pathogen-agnostic detection within 24–48 hours, potentially reducing unnecessary antibiotic exposure.[62–64] While its high cost (3900 RMB/test) and interpretation complexities remain barriers, these may be offset by shortened ICU stays and targeted therapy initiation. Conversely, in resource-limited regions, mNGS offers strategic value in outbreak settings and for culture-negative fever syndromes, circumventing infrastructure gaps through centralized sequencing hubs.[65] Key advantages including unbiased pathogen detection and resistance gene profiling must be balanced against challenges: bioinformatics dependence in high-income systems, and electricity/equipment stability constraints in low-income areas.

The study has several limitations. Firstly, it is a single-center, retrospective study, which introduces uncontrollable variables such as the timing of mNGS detection and prior anti-infective treatments. Secondly, the small sample size in each group, particularly in the fungal infection cohort, was inadequate. Further research with a larger number of participants is needed to better understand the microbiota identified in this preliminary study. Additionally, most CNS infection diagnoses were based on clinical findings rather than definitive pathogenic evidence, which leaves room for potential misclassification despite our rigorous efforts.

Conclusion

The application of mNGS to CNS infection analysis facilitates comprehensive pathogen detection across broad taxonomic ranges. Subsequent studies will expand our patient cohort to establish a more robust microbial atlas of intracranial infections, ultimately enabling enhanced diagnostic precision and deeper clinical insights mNGS analysis of CNS infections offers a broader pathogen detection spectrum, leading to more accurate diagnoses and deeper clinical insights.

Ethics Approval

This study received ethical approval from the the institutional review board and ethics committee of the First Affiliated Hospital of Fujian Medical University (MRCTA, ECFAH of FMU [2024]459) and was conducted in accordance with the principles outlined in the *Declaration of Helsinki*.

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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 no competing interests in this work.

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