ORIGINAL RESEARCH

Diagnostic Performance of Metagenomic Next-Generation Sequencing in Central Nervous System Cryptococcosis Using Cerebrospinal Fluid

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Purpose: Metagenomic next-generation sequencing (mNGS) has been widely used to diagnose infectious diseases. However, there are few studies on its diagnostic performance in the central nervous system (CNS) cryptococcosis. This study examined the diagnostic efficacy of mNGS in identifying *Cryptococcus* spp. in cerebrospinal fluid (CSF) samples.

Patients and Methods: From March 2021 to March 2023, 290 patients with suspected CNS infection were recruited from the First Affiliated Hospital, School of Medicine, Zhejiang University, and 74 patients were ultimately included in the study. Lastly, 22 patients with CNS cryptococcosis were included. Of these patients, 25 CSF samples were enrolled. The diagnostic performance of conventional assays [including India ink, cryptococcal antigen (CrAg) testing, and culture] and mNGS was evaluated for CNS cryptococcosis.

Results: In the 25 samples collected, the coincidence rates of mNGS with India ink, CrAg, and culture were 64.0% (16/25), 80.0% (20/25), and 80.0% (20/25), respectively. Without antifungal drug exposure, the coincidence rates were increased to 66.7% (10/15), 100.0% (15/15), and 93.3% (14/15), respectively. The coincidence rates after antifungal therapy were all decreased to 60.0% (6/10), 50.0% (5/10), and 60.0% (6/10), respectively. Moreover, in the 25 samples, the sensitivity of mNGS reached 80.0%, and of India ink, CrAg testing, and culture were 68.0, 100.0, and 60.0%, respectively. The mNGS showed an excellent positive rate (100.0%) in the 15 samples collected without antifungal drug exposure, which was significantly higher than the antifungal drug-exposed group (n = 10) (50.0%) (*P* = 0.005). The reads of *Cryptococcus* spp. before antifungal therapy were significantly higher than after it (median, 25,915 vs 2, *P* = 0.008).

Conclusion: mNGS is an effective tool for diagnosing CNS cryptococcosis using CSF; however, its sensitivity decreases considerably in patients who have been effectively treated with antifungal drugs.

Keywords: metagenomic next-generation sequencing, central nervous system cryptococcosis, cerebrospinal fluid, invasive fungal infection

Introduction

Central nervous system (CNS) cryptococcosis is an invasive fungal infection caused by *Cryptococcus* spp. Although the infection is preventable and treatable, its morbidity and mortality rates are still high.[1] Global cryptococcal meningitis accounts for 15% of acquired immune deficiency syndrome-related deaths, with an estimated 181,100 deaths annually.[2] In the Asia and Pacific region, approximate 44,000 HIV-infected adults developed cryptococcal meningitis, and 26,000 cryptococcal-related deaths occurred annually.[3] Consequently, effective prevention, prompt and accurate diagnosis, and early treatment are critical.

Traditional diagnostic methods of CNS cryptococcosis include cerebrospinal fluid (CSF) culture, India ink, and cryptococcal antigen (CrAg) testing. Among them, CSF culture is considered the diagnostic gold standard.[2] However, it takes several days, or even a week, for fungus to develop, which can delay diagnosis and treatment. Cultures may also produce false-negative results when the fungal burden is low.[4] India ink microscopy has historically been a rapid and

low-resource technique for detecting *Cryptococcus* spp. in the CSF.[5] It demonstrates relatively low sensitivity (86% in cryptococcal meningitis)[6] and is technician-dependent.[7,8] CrAg testing was an effective method to detect cryptococcosis, which was more sensitive than CSF culture.[9] The summary estimates of sensitivity and specificity of CSF CrAg for detecting cryptococcal meningitis were 98.8 and 99.3%, respectively, and summary sensitivity and specificity of serum CrAg were 99.7% and 94.1%, respectively.[10]

Metagenomic next-generation sequencing (mNGS) is a method where all the nucleic acid (DNA and/or RNA) of a specimen is sequenced in parallel, which is highly effective in diagnosing infectious diseases.[11–13] Previous studies[14–16] demonstrated that, in some cases, mNGS improved the diagnosis of neurologic infections and provided actionable information on CSF obtained from patients with meningitis. However, only a few studies have focused on CNS cryptococcosis.[17–19] Therefore, this retrospective study evaluated the diagnostic performance of mNGS in identifying CNS cryptococcosis compared to traditional culture, India ink, and CrAg testing of CSF.

Materials and Methods
Study Design and Participants
A retrospective observational study was performed in patients with suspected CNS infections from March 2021 to March 2023. The inclusion criteria of patients were as follows: (1) patients aged over 18 years; (2) patients who visited the First Affiliated Hospital, School of Medicine, Zhejiang University, from March 2021 to March 2023; (3) patients had symptoms of meningitis or encephalitis, including fever, headache, nausea/vomiting, seizures, alteration in consciousness/ coma; and (4) patients received lumbar puncture for CSF test, included mNGS, culture, India ink, and CrAg testing. Exclusion criteria were: (1) patients with incomplete clinical data; (2) patients without mNGS, culture, India ink, or CrAg testing on CSF. The diagnosis of CNS cryptococcosis was based on the guideline of European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (2019).[20] The enrollment and screening process of patients is depicted in Figure 1.

Data were extracted from the electronic patient record system of the hospital. Demographic characteristics (age and gender), comorbidities, laboratory results (CSF routine test, biochemical tests, mNGS, culture, India ink, and CrAg testing), clinical manifestations, intensive care unit treatment history, and ventriculoperitoneal shunts were collected.

Culture, India Ink, and CrAg Testing
All samples were cultured on Sabouraud dextrose agar (SDA) at 37°C for 2–5 days. The growing colonies were purified for culture, and a single colony was selected for strain identification using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). *Cryptococcus neoformans* was shown as a yeast-like fungus with a thick, clear capsule through Indian ink staining. CSF-based CrAg lateral flow immunoassay tests (Immy Diagnostics, Norman, Oklahoma, USA) were performed on each sample according to the manufacturer's instructions.

mNGS on CSF Samples
CSF mNGS testing was performed in the clinical laboratory of our hospital. The steps included sample processing, nucleic acid extraction, library construction, high-throughput sequencing, and bioinformatics analysis (result interpretation). First, total DNA was extracted from 300 μL of CSF samples using a commercial kit (QIAamp® UCP Pathogen DNA Kit, catalog number: 50,214, Qiagen, Germany). Each sample was spiked with an internal control after nucleic acid extraction in a manner described in detail in our recently published study.[21] Subsequently, 30 μL of the nucleic acid solution was used to generate a sample library using a commercial library construction kit (Nextera DNA Flex kit, Illumina, San Diego, CA, USA). The synthesized library was single-end sequenced (SE-75) using an Illumina Nextseq CN500 sequencer. In each run, negative and no-template control samples were tested alongside clinical samples to identify potential test failures and microbial contaminations. After sequencing, we used the same analysis software and algorithms as in our published studies to filter the raw data into clean data, then remove the human sequence by mapping the clean data to the human reference genome (hg38), and the remaining non-human sequence are finally aligned to the microbial reference database to complete the identification of microorganisms.[21]
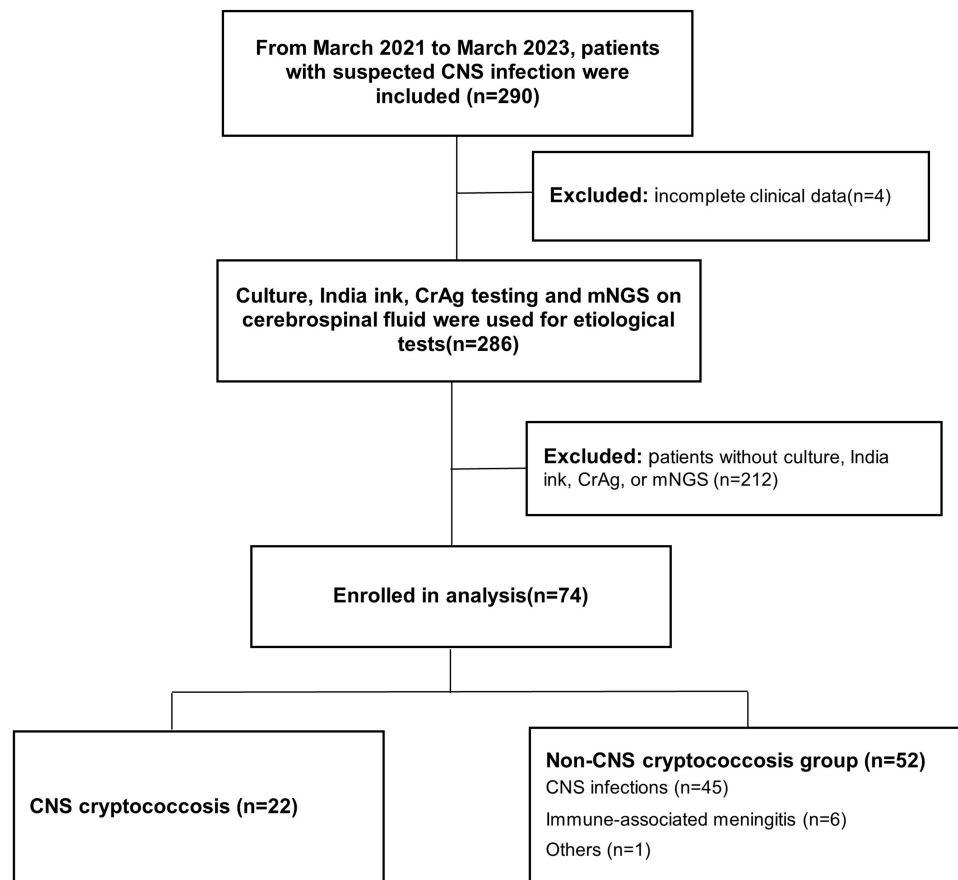
Figure 1 Flowchart of patient enrollment and grouping (CNS, central nervous system; CrAg, cryptococcal antigen; mNGS, metagenomic next-generation sequencing).

Statistical Analysis

All statistical analyses were conducted with SPSS software version 25.0 (IBM, Armonk, NY, USA). Continuous variables with a normal distribution were expressed as mean ± standard deviation (SD), while continuous variables with a non-normal distribution were represented as median. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the Youden index were used to evaluate the diagnostic performance of India ink, CrAg, culture and mNGS for CNS cryptococcosis. The χ2 test was used to compare categorical data obtained from various tests. The reads of *Cryptococcus* spp. between various antifungal drug exposure states were analyzed by the Mann–Whitney *U*-test. $P < 0.05$ was considered to be statistically significant. Figures were drawn using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) and online website (https://bioinfogp.cnb.csic.es/tools/venny/index.html).

Results
Patient Characteristics

As displayed in Figure 1, 290 patients with suspected CNS infections were selected from March 2021 to March 2023. However, 212 patients were excluded due to the absence of CSF sample analysis (including mNGS, culture, India ink, and CrAg testing), and four patients were excluded due to incomplete clinical data. Consequently, 74 patients were ultimately included in the study. Based on the CSF sample analysis results, the patients were divided into the CNS cryptococcosis group (22 patients) and the non-CNS cryptococcosis group (52 patients). Three of the 22 patients with CNS cryptococcosis received CSF tests twice (including mNGS). A total of 25 CSF samples were collected. The mNGS and culture results suggested that all samples contained *Cryptococcus neoformans*.

Table 1 enlists the patient characteristics of the CNS cryptococcosis group and non-CNS cryptococcosis group. In CNS cryptococcosis group, the number of immunocompromised patients ($P = 0.003$) and patients with ventriculoperitoneal shunts

Table 1 Clinical Features of Patients with CNS Cryptococcosis and Non-CNS Cryptococcosis

Characteristics	CNS Cryptococcosis (n=22)	Non-CNS Cryptococcosis (n=52)	P value
Gender (n, %)			0.638
Male	14(63.6%)	36(69.2%)	–
Female	8(36.4%)	16(30.8%)	–
Age, years	49.64±17.58	45.35±15.79	0.152
Immunocompromised patient (n, %)	10(45.5%)	6(11.5%)	0.003
HIV	6(27.3%)	1(1.9%)	0.614
Liver transplantation	1(4.5%)	0(0.0%)	0.297
Kidney transplantation	1(4.5%)	0(0.0%)	0.297
Connective tissue diseases	2(9.1%)	1(1.9%)	0.197
Hematologic malignancy	0(0.0%)	2(3.8%)	0.491
Other application of immunosuppressants	0(0.0%)	2(3.8%)	0.491
Symptoms			
Fever	17(77.3%)	46(88.5%)	0.379
Headache	19(86.4%)	46(88.5%)	0.801
Nausea/vomiting	8(36.4%)	19(36.5%)	0.989
Seizure	6(27.3%)	11(21.2%)	0.567
Alteration in consciousness/coma	10(45.5%)	23(44.2%)	0.923
Meningitis	13(59.1%)	27(51.9%)	0.572
Meningoencephalitis	9(40.9%)	25(48.1%)	–
ICU treatment (n,%)	7(31.8%)	7(13.5%)	0.129
Ventriculoperitoneal shunts	11(50.0%)	2(3.8%)	<0.001

Abbreviations: CNS, central nervous system; HIV, human immunodeficiency virus; ICU, intensive care unit.

($P < 0.001$) increased significantly. Of the 25 CSF samples, 15 (60.0%) cases were collected without antifungal therapy exposure, while 10 (40.0%) were under antifungal therapy. The CSF assays in various stages of antifungal treatment are shown in Table 2, and none of them differed significantly ($P > 0.05$).

The Results of mNGS and Conventional Tests for *Cryptococcus* Spp.

The results of mNGS and conventional tests (India ink, CrAg, and culture) in the 25 CSF samples collected are shown in Figure 2A. The 11 samples were diagnosed positive by all the methods, however, seven samples were positive by three methods [4 samples (CrAg, culture, mNGS), 3 samples (India ink, CrAg, mNGS)]. Five samples were identified as positive by two distinct methods [2 samples (CrAg, mNGS), 3 samples (India ink, CrAg)]. Only two samples tested positive using a single method (CrAg).

Before antifungal drug exposure, ten of fifteen samples (10/15) were positive by all methods, four by three methods (CrAg, culture, mNGS), and one by CrAg and mNGS (Figure 2B). Under antifungal drug exposure, one sample was positive by all methods, three samples were positive by three methods (India ink, culture, mNGS), four samples were

Table 2 The Cerebrospinal Fluid Tests for Central Nervous System Cryptococcosis in Various Antifungal Treatment States

|	Without Antifungal Drug Exposure n = 15	Antifungal Drug Exposure n = 10	P value
CSF pressure (mm H$_2$O)	241.67±32.01	213.5±34.84	0.723
CSF test			
Karyocyte, μL	24.00	57.50	0.935
Protein, g/L	1.33±0.35	2.37±1.51	0.461
Chlorine, mmol/L	117.00	117.50	0.794
Glucose, mmol/L	2.70	2.60	0.683

Abbreviation: CSF, cerebrospinal fluid.

positive by two methods [1 samples (CrAg, mNGS), 3 samples (India ink, CrAg)], and two samples were positive by CrAg (Figure 2C).

Comparison of the Detection Capabilities of mNGS and Conventional CNS Cryptococcosis Tests

Table 3 displays the diagnostic efficacy of India ink, CrAg, culture, and mNGS using CSF in CNS cryptococcosis. The detection efficiency of mNGS for the 25 collected samples reached 80.0%, and the results of the four detection methods differed statistically ($\chi2 = 12.818$, $P = 0.005$). Among the 15 samples collected without antifungal drug exposure, mNGS and CrAg showed excellent detection efficiency (100.0%), and culture also had 93.3% detection efficiency, which was significantly higher than India ink (66.7%, $\chi2 = 12.593$, $P = 006$). However, mNGS (50.0%) did not perform than CrAg and India ink in antifungal drug exposure samples (100.0% vs 70.0%), and culture was the worst (10.0%, $\chi2 = 17.494$, $P = 0.001$).

The coincidence rates of mNGS with India ink, CrAg, and culture in 25 CSF samples were 64.0% (16/25), 80.0% (20/25), and 80.0% (20/25), respectively. Without antifungal drug exposure, the coincidence rates of mNGS with India ink, CrAg, and culture were increased to 66.7% (10/15), 100.0% (15/15), and 93.3% (14/15), respectively. The coincidence rates of mNGS with India ink, CrAg, and culture after antifungal therapy were all decreased to 60.0% (6/10), 50.0% (5/10), and 60.0% (6/10), respectively.
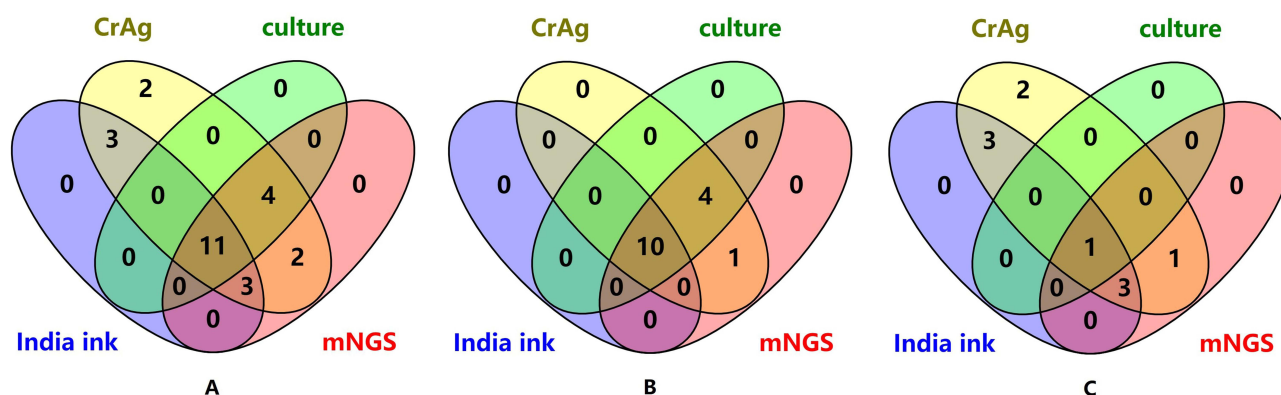
Figure 2 The Venn diagram of results of metagenomic next-generation sequencing (mNGS) and conventional tests [including India ink, cryptococcal antigen (CrAg) testing, and culture] in central nervous system cryptococcosis. Figure (**A**) depicts the results of all 25 CSF samples, Figure (**B**) shows the results of 15 CSF samples collected before antifungal drug exposure, and Figure (**C**) depicts the results of 10 samples collected after antifungal drug exposure.

Table 3 Diagnostic Performance of India Ink, CrAg, Culture, and mNGS in Central Nervous System Cryptococcosis

	Sensitivity	**Specificity**	**PPV**	**NPV**	**Youden Index**
Cryptococcus group (n=25)					
India ink	68.0%(17/25)	100.0%(52/52)	100.0%(17/17)	86.7%(52/60)	68.0%
Culture	60.0%(15/25)	100.0%(52/52)	100.0%(15/15)	83.9%(52/62)	60.0%
CrAg	100.0%(25/25)	100.0%(52/52)	100.0%(25/25)	100.0%(52/52)	100.0%
mNGS	80.0%(20/25)	100.0%(52/52)	100.0%(20/20)	91.2%(52/57)	80.0%
Antifungal drug exposure (n=10)					
India ink	70.0%(7/10)	100.0%(52/52)	100.0%(7/7)	94.5%(52/55)	70.0%
Culture	10.0%(1/10)	100.0%(52/52)	100.0%(1/1)	85.2%(52/61)	10.0%
CrAg	100.0%(10/10)	100.0%(52/52)	100.0%(10/10)	100.0%(52/52)	100.0%
mNGS	50.0%(5/10)	100.0%(52/52)	100.0%(5/5)	91.2%(52/57)	50.0%
Without antifungal drug exposure (n=15)					
India ink	66.7%(10/15)	100.0%(52/52)	100.0%(10/10)	91.2%(52/57)	66.7%
Culture	93.3%(14/15)	100.0%(52/52)	100.0%(14/14)	98.1%(52/53)	93.3%
CrAg	100.0%(15/15)	100.0%(52/52)	100.0%(15/15)	100.0%(52/52)	100.0%
mNGS	100.0%(15/15)	100.0%(52/52)	100%(15/15)	100.0%(52/52)	100.0%

Abbreviations: CrAg, cryptococcal antigen; mNGS, metagenomic next-generation sequencing; PPV, positive predictive value; NPV, negative predictive value.

Effect of Antifungal Drug Exposure on *Cryptococcus* Spp. Detection Using mNGS

There was a significant difference in the positive detection rate by mNGS between CNS cryptococcosis patients receiving antifungal therapy and those not receiving treatment (100.0 vs 50.0%, P = 0.005). However, in patients receiving antifungal drugs for > 3 months, the rate of *Cryptococcus* spp. detection by mNGS was lower than in cases receiving drugs for < 3 months [60.0% (3/5) vs 40.0% (2/5)]. Moreover, there was no statistical difference *(P = 0.573)*. Antifungal regimens and duration of antifungal therapy in patients with CNS cryptococcosis were detailed in Table S1.

Of the 25 CSF samples, the reads of *Cryptococcus* spp. before antifungal therapy were significantly higher than after it (median, 25,915 vs 2, P = 0.008) (Figure 3). Among the samples with antifungal drug exposure, two samples had only one read, and one had two reads. Three patients underwent CSF testing before and after antifungal therapy. According to
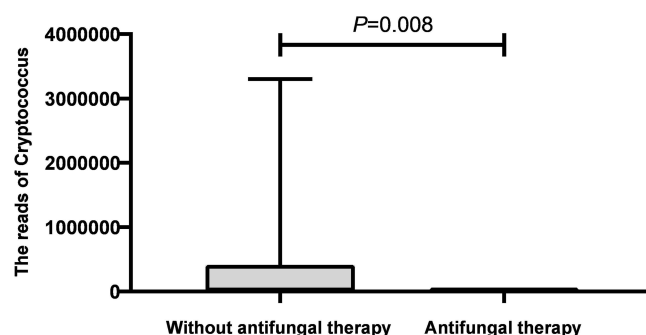
Figure 3 Comparison of *Cryptococcus* spp. reads measured by metagenomic next-generation sequencing in the cerebrospinal fluid of patients with central nervous system cryptococcosis with or without antifungal drug exposure. The difference was statistically significant (P = 0.008).

mNGS, the sequence of *Cryptococcus* spp. decreased significantly after antifungal treatment of two cases was undetectable, and that of one case was significantly reduced (217,218 vs 2060).

Discussion

Our study enrolled 22 patients with CNS cryptococcosis and 25 CSF samples. Some samples were collected after antifungal treatment, resulting in intriguing variations in the results. There was no significant difference in the routine tests of CSF before and after antifungal therapy. The reason may be that mNGS was examined to confirm the diagnosis again due to poor response to antifungal treatment or suspicion of a mixed infection.

Tests used in CNS cryptococcosis, an exceedingly severe disease with a high fatality, must be highly sensitive to ensure the timely initiation of induction antifungal therapy.[22] We evaluated the diagnostic performance of conventional tests (India ink, CrAg testing, and culture) and mNGS in detecting CNS cryptococcosis using CSF. The sensitivity of mNGS was slightly lower than CrAg testing, however, higher than India ink and culture; its specificity was identical to conventional methods. Interestingly, before antifungal drug exposure, the sensitivity of mNGS and CrAg reached 100.0%, superior to India ink and culture. It indicated excellent diagnostic efficacy of mNGS in CNS cryptococcosis using CSF. Similar to our findings, Gan Z et al[18] reported that in 46 cases of cryptococcal meningitis in HIV-negative patients, the sensitivity and specificity of mNGS reached 93.5 and 96.0%, respectively.

The role of CrAg testing in cryptococcosis diagnosis has been established by guidelines.[20,23] In this study, the CrAg test performed well, and its sensitivity in CSF reached 100.0% regardless of antifungal drug exposure. It is well known that antifungal drug exposure can significantly alter the results of conventional etiological tests because a decrease in cryptococcal load and cryptococcal activity may result in negative fungal culture and India ink staining results. However, it is unclear whether exposure to antifungal drugs affects the results of mNGS. Our study provided a conclusive answer. After antifungal treatment, the detection rate of *Cryptococcus* spp. with mNGS decreased from 100.0 to 50.0% ($P = 0.005$), even lower than CrAg and India ink and higher than culture. With the extension of antifungal drug application time, the rate of mNGS detection decreased. In addition, *Cryptococcus* spp. reads decreased substantially following antifungal therapy, confirming the efficacy of antifungal therapy. Therefore, antifungal therapy can considerably reduce the detection rate and reads of *Cryptococcus* spp. using mNGS detection. The cost of mNGS was expensive, and the time performance was not as good as India ink and CrAg testing. Therefore, mNGS was no longer suitable as a diagnostic tool for CNS cryptococcosis after antifungal drug exposure, with a significant decrease in sensitivity.

Our research has several limitations. First, this retrospective, single-center study had a limited sample size, which may have introduced statistical bias. Therefore, a large-scale multicenter prospective study is warranted to further elucidate the diagnostic utility of mNGS in CNS cryptococcosis. Second, the study's conclusion may be influenced by the inconsistent duration of antifungal exposure during sample collection. Consistent sampling intervals are essential for future studies. Furthermore, CrAg was only tested qualitatively, however, the specific titer was unclear.

Conclusion

The diagnostic performance of conventional tests (including India ink, CrAg testing, and culture) and mNGS was evaluated for detecting CNS cryptococcosis using CSF. The mNGS was less sensitive than CrAg, however, it was more sensitive than India ink and culture. Before antifungal drug exposure, mNGS and CrAg sensitivity reached 100.0%. After exposure to antifungal drugs, the *Cryptococcus* spp. detection rate and reads using mNGS significantly decreased, and the sensitivity of mNGS were worse than CrAg and India ink. Therefore, mNGS is an effective tool for diagnosing CNS cryptococcosis using CSF; however, its sensitivity decreases considerably in patients effectively treated with antifungal drugs, CrAg and India ink may be the better detection methods than mNGS.

Data Sharing Statement

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

Ethics Approval and Informed Consent

This investigation was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University. The procedures used in this study adhere to the tenets of the Declaration of Helsinki. Because this was a retrospective observational study, informed consent to participate was waived in accordance with the ethics approval. The patients' privacy will be kept strictly confidential.

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 report no conflicts of interest in this work.

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