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Comparison of mNGS with conventional methods for diagnosis of cryptococcal meningitis: a retrospective study

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The application of metagenomic next-generation sequencing (mNGS) in the diagnosis of cryptococcal meningitis is relatively under characterized. Here, we retrospectively evaluated data from cryptococcal meningitis patients who were tested using mNGS and/or routine testing, including fungal culture, India ink staining, and cryptococcal antigen (CrAg) testing. The performance of mNGS was then assessed. Initial cerebrospinal fluid (CSF) samples were collected from 65 patients with suspected central nervous system (CNS) infection and tested using conventional tests and/or mNGS. mNGS offers a culture-independent approach, facilitating a rapid and unbiased detection of a broad spectrum of pathogens. Patients with bacterial tuberculous or viral meningitis were used as mNGS-positive controls and one autoimmune encephalitis patient was used as an mNGS-negative control. In the 45 patients diagnosed with cryptococcal meningitis, the sensitivity, specificity, positive predictive value, negative predictive value, and concordance rate of mNGS were 92%, 100%, 100%, 90.9%, and 95.6%, respectively. Compared to conventional methods, the sensitivity of mNGS was slightly lower than CrAg tests (96.7%) but higher than India ink (79.5%) and culturing (63.4%). Of the two negative mNGS cases (2/25, 8.0%), one was positive by India ink staining, culture, and CrAg testing, while the other was positive only by CrAg testing. A combination of mNGS and conventional methods enhanced the detection rate to 100%. Our study demonstrates that both CrAg and mNGS offer excellent diagnostic accuracy for cryptococcal meningitis, and utilizing both tests can enhance clinical assessment and patient management.

Keywords Metagenomic next-generation sequence, Conventional method, Cryptococcal meningitis, Diagnosis, Cerebrospinal fluid

Cryptococcal meningitis is a high-morbidity manifestation of cryptococcosis, which is caused by ubiquitous basidiomycete yeasts *Cryptococcus*[1,2]. *Cryptococcus neoformans* (*C. neoformans*) and *Cryptococcus gattii* (*C. gattii*) are two major pathogenic cryptococci leading to life-threatening cryptococcal meningitis[3]. Cryptococcal meningitis is primarily associated with immunocompromised individuals, with human immunodeficiency virus (HIV) infection often being correlated with its occurrence. Most HIV-associated cryptococcal meningitis occurs in patients with cluster of differentiation 4 (CD4) cell count < 200 cells/mm^3 and is frequently the cause of death in patients[4]. Apart from HIV, other factors that lead to immune suppression, such as diabetes, liver cirrhosis, renal failure, and patients with long-term use of steroids therapy or other immunosuppressive agents, also render patients susceptible to *Cryptococcus*[5]. Collectively, this group of immunosuppressive individuals and immunocompetent individuals are referred to as HIV-negative cryptococcal meningitis patients. There is an increasing number of cases where HIV-negative individuals are infected by *Cryptococcus*[6]. To exacerbate the

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No.	Sex	Age	Underlying diseases	SD	Main complaint	*P* (mmH_2O)	WBC (/μL)	PMC (%)	Glucose (mmol/L)	Protein (g/L)	IIS	Cul	CrAg	mNGS	Final diagnosis
1	M	48	Viral Hepatitis type B	12	Recurrent headaches	290	786	84.7	5.16	1.14	-	+	+	NA	Cryptococcal meningitis
2	F	23	-	10	Headache with fever and vomiting	> 330	55	NA	2.53	0.47	+	NA	+	NA	Cryptococcus neoformans meningitis
3	M	56	Sjogren syndrome	90	Recurrent headaches	160	167	NA	1.83	>3	+	-	+	NA	Cryptococcal meningitis
4	M	59	Cerebral infarction	20	Headache with vomiting	NA	115	68.7	1.2	2.39	+	+	NA	NA	Intracranial infection (cryptococcal and suppurative meningitis)
5	F	48	-	30	Headache	> 330	187	NA	<1.11	1.62	+	NA	NA	NA	Cryptococcus neoformans meningitis
6	F	52	-	6	Recurrent headaches	190	113	95.6	95.6	0.46	+	-	NA	NA	Cryptococcal meningitis
7	F	55	-	30	Vertigo and unstable walking	130	53	86.8	<1.11	>3	NA	+	NA	NA	Cryptococcal meningitis
8	M	52	Hyperlipidemia	15	Headache, double vision and hearing loss	320	80	93.8	1.2	0.73	+	+	NA	NA	Cryptococcal meningitis
9	M	55	Hypothyroidism	30	Recurrent headache	>330	240	60	2.33	0.94	+	+	NA	NA	Cryptococcal meningoencephalitis
10	F	74	Diabetes, hypertension	30	Headache	>330	201	98.5	2.08	1.05	+	+	NA	NA	Cryptococcus neoformans meningitis
11	F	60	Rheumatoid arthritis, cerebral infarction	60	Recurrent headaches	310	120	99.2	2.02	1.68	-	+	+	+	Cryptococcus neoformans meningitis
12	M	62	Obsolete tuberculosis	17	Recurrent headache with low-grade fever	245	35	77.1	3.27	0.27	+	-	NA	NA	Cryptococcal meningoencephalitis
13	F	71	Obsolete tuberculosis, hypertension	15	Headache	70	38	100	3.18	0.47	+	-	NA	NA	Cryptococcal meningitis
14	M	58	Silicosis	10	Headache	190	295	60.3	1.13	2.89	+	-	NA	+	Cryptococcal meningoencephalitis
15	M	45	Fatty liver	30	Headache	300	339	92.3	1.66	1.02	+	+	+	NA	Cryptococcal meningoencephalitis
16	M	46	Ankylosing spondylitis	3	Fever with headache	NA	21	61.9	1.48	1.57	+	+	+	NA	Cryptococcal meningitis
17	M	62	Cerebral infarction, hypertension	30	Recurrent headaches	>330	181	69.6	<1.11	1.28	+	NA	+	NA	Cryptococcal meningitis
18	M	48	-	20	Dizziness and headache	180	29	96.6	4.19	0.77	+	-	NA	NA	Cryptococcal meningitis
19	M	51	Diabetes	8	Involuntary twitching of the face and limbs with headache	300	99	59.6	3.06	2.26	+	+	NA	NA	Cryptococcal meningitis
20	M	38	Cerebral infarction	60	Fever, headache, bilateral droopy eyelids with double vision	100	311	96.8	2.15	1.51	-	-	NA	+	Cryptococcal meningitis
21	M	40	AIDS	4	Headache	>330	8	NA	2.72	0.35	+	NA	NA	NA	Cryptococcal meningitis
22	M	71	-	10	Headache	>330	153	97.4	<1.11	0.76	+	+	+	+	Cryptococcal meningitis
23	M	66	Myasthenia Gravis	10	Fever and headache	180	140	42.9	1.37	1	+	+	+	+	Cryptococcus neoformans meningitis
24	M	44	Hyperlipidemia	8	Fever and headache	>330	302	84.1	2.51	0.8.	-	+	+	+	Cryptococcal meningitis
25	F	66	Autoimmune hemolytic anemia, diabetes	7	Slurred speech, headache and unconsciousness	270	18	100	5.26	0.81	+	-	+	+	Cryptococcal meningitis
26	M	47	-	14	Recurrent fever with headache	270	91	95.6	<1.11	1.18	+	+	+	+	Cryptococcal meningitis
27	F	67	Hypertension	10	Fever with headache	100	104	100	2.2	0.75	+	-	+	+	Cryptococcal meningitis
28	F	36	Nephrotic syndrome	7	Recurrent headaches	>330	419	77.1	<1.11	>3	+	+	+	+	Cryptococcal meningitis
29	M	40	Hypertension, hyperlipidemia	60	Headache	200	323	99.1	1.6	1.04	+	+	+	+	Cryptococcal meningitis
30	F	59	-	60	Fever with headache	260	37	59.5	2.69	0.73	+	-	+	+	Cryptococcal meningitis
31	M	73	-	90	Headache with fever	>330	129	86.8	0.97	0.62	+	+	+	+	Cryptococcal meningitis
32	M	62	Cerebral infarction	60	Headache with mental abnormality	120	26	100	3.95	0.54	-	-	+	+	Cryptococcal meningoencephalitis
33	M	39	-	14	Headache	280	133	100	1.47	0.77	+	-	+	+	Cryptococcal meningitis
34	F	72	Obsolete tuberculosis, anemia	10	Headache and weakness	60	98	85.7	1.89	0.72	+	+	+	-	Cryptococcal meningitis
35	F	49	Sjogren syndrome	60	Recurrent headache with vomiting	190	77	77.9	1.35	1.09	-	+	+	+	Cryptococcal meningoencephalitis
36	M	61	-	7	Fever, headache and unresponsiveness	160	110	100	2.16	2.08	-	-	+	+	Cryptococcal meningoencephalitis
37	F	40	Systemic lupus erythematosus	90	Headache, fever and dizziness	280	21	100	1	0.65	+	+	+	+	Cryptococcal meningitis

Continued

No.	Sex	Age	Underlying diseases	SD	Main complaint	*P* (mmH_2O)	WBC (/μL)	PMC (%)	Glucose (mmol/L)	Protein (g/L)	IIS	Cul	CrAg	mNGS	Final diagnosis
38	M	64	-	60	Headache with dizziness and double vision	80	81	96.3	2.22	1.18	-	-	+	-	Cryptococcal meningitis
39	M	65	-	30	Recurrent fever	80	225	87.1	3.92	0.93	+	+	+	+	Cryptococcal meningitis
40	M	73	-	60	Recurrent dizziness	190	12	91.7	2.11	0.98	-	+	-	+	Cryptococcal meningitis
41	F	59	Dermatomyositis, diabetes	5	Headache and weakness of limbs	250	39	46.2	5.64	0.64	+	+	+	NA	Cryptococcal meningitis
42	F	50	-	7	Headache	120	186	99.5	2.95	0.41	+	+	+	+	Cryptococcal meningitis
43	M	27	-	10	Recurrent headaches	>330	292	74	2.08	0.8	+	+	+	+	Cryptococcal meningoencephalitis
44	M	65	-	18	Headache	>330	30	86.7	2.85	0.25	+	+	+	+	Cryptococcal meningitis
45	M	56	Viral Hepatitis type B	10	Recurrent fever and headache	210	177	88.7	1.61	0.87	+	-	+	NA	Cryptococcus neoformans meningitis
46	M	73	Hypertension	40	Cough and sputum	270	2007	27.7	3.32	1.89	-	-	+	-	Purulent meningitis
47	M	56	Hypertension, Hyperlipidemia	30	Recurrent fever with headache	>330	148	95.9	2.26	1.07	-	-	-	-	Tuberculous meningoencephalitis
48	M	60	Cerebral infarction	25	Headache	70	435	46	1.29	>3	-	-	-	-	Tuberculous meningoencephalitis
49	F	38	-	330	Weakness of both lower limbs	130	7	85.7	2.11	>3	-	-	-	-	Tuberculous meningitis
50	F	67	Neurosyphilis	10	Headache with fever	60	304	99	1.33	>3	-	-	-	-	Tuberculous meningitis
51	M	51	Hypertension, metabolic arthritis,	2	Slow reaction, dizziness and vomiting	>300	396	98.5	1.7	2.28	-	-	-	-	Tuberculous meningitis
52	M	20	-	14	Cough, sputum and impaired consciousness	265	286	28.3	2.36	1.18	-	-	-	-	Tuberculous meningitis
53	F	72	Hypertension, rheumatoid arthritis	3	Slow response	220	53	83	1.73	1.72	-	-	-	-	Tuberculous meningitis
54	F	68	Cerebral infarction, diabetes	7	Headache and dizziness with weakness	165	142	85.2	1.87	1.04	-	-	-	-	Tuberculous meningitis
55	M	38	Diabetes	240	Recurrent headache with fever	210	102	97.1	2.24	1.88	-	-	-	-	Tuberculous meningitis
56	M	40	-	7	Headache	>330	116	99.1	2.68	1.8	-	-	-	-	Tuberculous meningitis
57	F	57	-	60	Dizziness with weakness of both lower limbs	90	337	99.7	2.82	4.24	-	-	-	-	Tuberculous meningitis
58	M	49	-	10	Fever with impaired consciousness	NA	343	98.8	3.23	4.05	-	-	-	-	Tuberculous meningitis
59	M	68	-	20	Headache	220	150	NA	1.39	3.28	-	-	-	-	Tuberculous meningitis
60	M	45	-	60	Headache	NA	265	20.6	2.42	2.82	-	-	-	-	Tuberculous meningitis
61	M	29	-	30	Fever and headache with left-sided facial twitching	360	70	NA	1.87	1.81	-	-	-	-	Tuberculous meningitis
62	M	46	-	17	Headache with fever	>330	308	90.3%	1.19	89.5	-	-	-	-	Tuberculous meningitis
63	F	53	-	4	Recurrent dizziness and headache, unresponsiveness	>300	389	84.3	1.66	2.51	-	-	-	-	Tuberculous meningoencephalitis

Continued

No.	Sex	Age	Underlying diseases	SD	Main complaint	*P* (mmH_2O)	WBC (/μL)	PMC (%)	Glucose (mmol/L)	Protein (g/L)	IIS	Cul	CrAg	mNGS	Final diagnosis
64	M	49	-	6	Headache with vomiting with abnormal behavior	115	33	100	4.63	0.77	-	-	-	-	Herpes simplex virus encephalitis
65	M	15	-	10	Abnormal mental behavior	190	44	100	2.95	0.23	-	-	-	-	Anti-NMDAR encephalitis

Table 1. Demographic data, CSF findings, diagnostic tests results and final diagnosis of participants. No. = case number; SD = symptom duration before admission (days); P = Intracranial pressure; WBC = white blood cell; PMC = percentage of mononuclear cell; IIS = India ink stain; Cul = culture; CrAg = cryptococcal antigen; mNGS = metagenomic next-generation sequence; M = male; F = female; NA = not available; - = negative; + = positive.

issue, the lack of specificity in early presentation of cryptococcal meningitis often leads to delayed diagnosis, resulting in a higher mortality rate in HIV-negative patients[7]. Despite the recognition of early diagnosis and targeted treatment as crucial factors in improving patient outcomes[8], the early detection of cryptococcal meningitis remains a significant challenge.

The conventional methods for detecting cryptococci include fungal culture, microscopic analysis of India ink staining smears, and cryptococcal antigen (CrAg) testing. While cryptococcal culture is the gold standard for the diagnosis of cryptococcal meningitis, the low detection rate and 3–5 days testing time both limit its use as an early diagnostic option[7]. India ink staining is a rapid method, but its sensitivity is reliant upon operator experience. CrAg testing has high sensitivity and specificity, but cannot determine the presence of infection, as it may remain positive for several weeks to months following the resolution of the disease. Additionally, it cannot detect antigen-deficient strains or distinguish pulmonary lesions[9,10]. A study evaluating the performance of the BioFire FilmArray Meningitis/Encephalitis (ME) Panel in detecting cryptococcus in CSF reported a sensitivity of 96% and specificity of 100% when the colony-forming unit (CFU) concentration exceeded 100/mL[11]. However, the sensitivity of detection is compromised when the fungal load is low[12]. Additionally, there have been reports of false-positive results associated with the use of the BioFire ME Panel[13]. Therefore, rapid, sensitive, and on-site detection methods are urgently required to accomplish cryptococcal meningitis diagnosis.

Metagenomic next-generation sequencing (mNGS) is capable of compensating for the shortcomings of the above techniques, featuring short reaction time, combined with high sensitivity and specificity, thus meeting the requirements of in-field cryptococcal meningitis diagnosis[10,14]. Recently, mNGS has emerged as an unbiased approach that can theoretically detect all pathogens in clinical samples. It is especially suitable for novel, rare, and atypical manifestations of infectious diseases[10,15]. As mNGS has the potential to identify any pathogen in CSF from patients with intracranial infection, it may be used as a front-line or second-line diagnostic tool for infectious meningitis, especially undiagnosed or chronic cases[16]. Previous studies on the use of mNGS for detecting cryptococcal meningitis have largely been limited to case reports or small-scale cohorts, often lacking validation in diverse clinical scenarios. This study expands on these findings by demonstrating the diagnostic value of mNGS, particularly its high sensitivity and its ability to identify complex cases such as mixed and ectopic infections. Here, we retrospectively evaluated 45 cryptococcal meningitis patients with both mNGS and conventional tests performed on CSF samples, attempting to assess performance of mNGS in the early diagnosis of cryptococcal meningitis. Moreover, we sought to illustrate the sensitivity and specificity among all methods.

Results

Clinical features of the participants

Among the 65 recruited patients, as illustrated in Table 1, the age of those diagnosed with cryptococcal meningitis ranged from 15 to 74 years, with a mean age of 54.5 years. Clinical features indicated that the majority were admitted approximately one week after symptom onset, with the duration of symptoms before admission ranging from 3 to 90 days. The median and interquartile range (IQR) of the duration of symptoms before admission were 15 and 20, respectively. Systemic symptoms included fever (14/45, 31.1%) and vomiting (3/45, 6.7%). The most common neurological symptom in patients with cryptococcal meningitis is headache (41/45, 91.1%), followed by other neurological manifestations: dizziness (5/45, 11.1%), double vision (3/45, 6.7%), and unconsciousness (3/45, 6.7%). Elevated intracranial pressure (ICP) was noted in a significant proportion of patients (24/43, 55.8%). CSF analysis revealed an increased white blood cell count, decreased glucose levels, and elevated protein concentration. Complications associated with central nervous system diseases include hydrocephalus (9/45, 20%) and neurological deficits (12/45, 26.7%). Of the neurological deficits observed, 3 cases (6.7%) were noted to develop during the course of therapy. Based on classification criteria and the detection of cryptococcal pathogens in the CSF samples[17], 45 patients met the criteria for a definite diagnosis of cryptococcal meningitis. Among 41 patients with positive ink staining and/or culture results, 35 patients were positive for ink staining, and 6 patients were diagnosed by India ink alone. None of these 6 patients underwent CrAg testing or mNGS. Notably, 4 of these 6 patients had negative cultures, while 2 patients did not undergo culturing. Among the remaining 4 patients, 1 was diagnosed solely through CrAg, 1 exclusively through mNGS, while the other 2 tested positive for both CrAg and mNGS. Follow-up antifungal therapy confirmed their cryptococcal infections. Among all cryptococcal meningitis patients, only patient 4 exhibited poor antifungal efficacy, defined as a lack of clinical improvement after a few days of appropriate antifungal treatment, worsening symptoms, and an increase in CSF white blood cell (WBC) count. Shortly thereafter, he completed a second lumbar puncture, which showed a cerebrospinal fluid WBC count of $6{,}050 \times 10^6$/L. His symptoms improved with simultaneous antifungal and antibacterial treatment. We therefore concluded that he was probably experiencing a mixed infection with both cryptococci and bacteria, despite the absence of conclusive bacteriological evidence. Overall, all cases in this study were caused by *C. neoformans*. In terms of immunocompromised status, 31 of the 45 (68.9%) patients were not immunocompromised, while 14 were, including 2 with Sjögren's syndrome and 1 with HIV/AIDS. Clinical and microbiological characteristics were similar in both immunocompromised and healthy patients. As controls, we also recruited one patient with purulent meningitis (Number 46), 17 patients with tuberculous meningitis (Numbers 47–63), one patient with herpes simplex virus encephalitis (Number 64), and one patient with autoimmune encephalitis (Number 65), with diagnostic tests performed on these patients as well. Information on all control patients is also presented in Table 1.

Identification of cryptococcal DNA in CSF samples by mNGS

Among the 25 cryptococcal meningitis cases tested by mNGS, stringently mapped reads to *Cryptococcus* spp. were identified in the CSF of 23 patients (23/25, 92%). The identified number of reads mapped to the *C. neoformans* sensu lato (s.l.) genome ranged from 2 to 306,606 (median 350) and are listed in Table 2. All the mapped reads

No.	species	reads
11	C.n	NA
14	C.n	NA
20	C.n	98
22	C.n	6950
23	C.n	NA
24	C.n	106
25	C.n	49,101
26	C.n	NA
27	C.n	2
28	C.n	NA
29	C.n	122
30	C.n	262
31	C.n	152,863
32	C.n	NA
33	C.n	350
35	C.n	43
36	C.n	730
37	C.n	4312
39	C.n	9
40	C.n	306,606
42	C.n	66
43	C.n	8146
44	C.n	2071
46	*Nocardia*	69
47	TB	12
48	TB	386
49	TB	119
50	TB	4
51	TB	16
52	TB	66
54	TB	1
55	TB	74
56	TB	2
57	TB	2
65	HSV	358

Table 2. Reads of positive mNGS cases. No. = case number; C.n = *Cryptococcus neoformans*; TB = *Tuberculosis*; HSV = *Herpes simplex virus*; NA = not available.

to *Cryptococcus* spp. were validated in the NT database on the NCBI by BLAST search. The identity of alignment to *Cryptococcus* spp. was greater than 90% in cases with positive mNGS.

Control cases 47 through 63 were diagnosed with a definite TBM based upon the classification criteria and evidence of MTB pathogens from at least one of mNGS, PCR, or Xpert MTB/RIF tests of the CSF samples[18]. mNGS found a different number of reads mapping to mycobacterial DNA as illustrated in Table 2. A very high number of unique reads mapped to *Nocardia* in case 46. qPCR and mNGS indicated positive identification of HSV1 in patient 64. Patient 65 was diagnosed with anti-NMDAR encephalitis based on detection of anti-NMDAR antibodies in his serum (1:100) and CSF (1:32) with no pathogens identified by mNGS in the CSF sample. No sequences of *Cryptococcus* spp. were identified in any control samples, thus, the specificity of mNGS to detect *Cryptococcus* spp. was 100% in this study.

Comparison of mNGS findings to conventional methods

Conventional methods used in this study included India ink staining, CrAg testing, and culturing of *Cryptococcus*. Culturing is the gold standard, however, the detection rate is low, as shown in Table 3. Among the 41 tested patients, only 26 patients were positive based upon fungal culture of CSF samples (26/41, 63.4%). These patients were also positive based upon India ink staining, CrAg, and mNGS tests except patient 7, who only had culturing performed, without enough CSF available for other tests. Thirty-five patients were positive based upon India ink staining tests of their first CSF samples (35/44, 79.5%). However, the CrAg test was available recently and only performed on the first CSF samples of 30 patients, with 29 cases identified positively by the CrAg method (29/30, 96.7%). The results of all four methods are shown in Fig. 1.

Method	Positive numbers	Numbers without testing	Detection rate
India ink examination	35	1	79.5(35/44)
Culture	26	4	63.4(26/41)
CrAg	29	15	96.7(29/30)
mNGS	23	20	92.0(23/25)

Table 3. The detection rate among mNGS and conventional methods for all recruited CM patients.

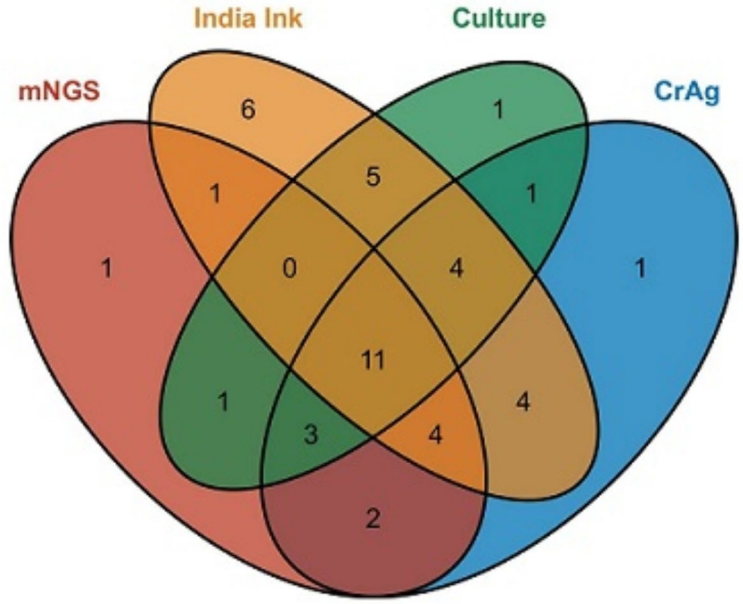

Fig. 1. Venn diagram of conventional cryptococcal testing and metagenomic next-generation sequencing (mNGS) results in 45 patients with central nervous system cryptococcal infections. The number of cases tested by India ink stain, culture, cryptococcal antigen (CrAg) testing and mNGS was 44, 41, 30 and 25, respectively, and the number of positive cases was 35, 26, 29 and 23, respectively.

Method	Sensitivity (%)	Specificity (%)	Positive predict value (%)	Negative predict value (%)	Accuracy (%)
India ink	79.5 (35/44)	100 (20/20)	100 (35/35)	69.0 (20/29)	85.9 (55/64)
culture	63.4 (26/41)	100 (20/20)	100 (26/26)	57.1 (20/35)	75.4 (46/61)
CrAg	96.7 (29/30)	95.0 (19/20)	96.7 (29/30)	95.0 (19/20)	96.0 (48/50)
mNGS	92.0 (23/25)	100 (20/20)	100 (23/23)	90.9 (20/22)	95.6 (43/45)

Table 4. Comparison of the sensitivity, specificity, positive predict value, negative predict value, and accuracy among four methods in patients with CM.

Unfortunately, due to the high cost of mNGS, only 25 cryptococcal meningitis cases were tested with mNGS using the first CSF samples. Interestingly, mNGS identified sequences mapping to cryptococcal DNA from 23 patients (23/25, 92.0%). The agreement of mNGS results with the gold standard culturing method was 60.0% (15/25), and its overlap with all conventional methods was 96.0% (24/25). Furthermore, the agreement of mNGS with the three conventional methods was 95.6% (43/45). As shown in Table 4, patients with cryptococcal meningitis had positive predictive and negative predictive values of cryptococcal meningitis by mNGS of 100% and 90.9%, respectively. The accuracy of mNGS was 95.6% in this study. The sensitivity of mNGS was significantly higher than that of culturing, the current gold standard (92.0% vs. 63.4%, $p = 0.03$). However, there was no significant difference between mNGS and CrAg testing (92.0% vs. 100%, $p = 1$) or India ink staining (92.0% vs. 79.5%, $p = 0.07$, respectively).

Regarding specificity, all four methods were 100% specific, with no significant differences observed among them.

Discussion

In the present study, we recruited 45 patients with a final diagnosis of cryptococcal meningitis and retrospectively evaluated the performance of mNGS for cryptococcal meningitis diagnosis. Compared with conventional diagnosis methods for the first CSF samples, the sensitivity of mNGS for the diagnosis of cryptococcal meningitis was slightly lower that of CrAg, but higher than that of India ink staining and culture. Combination of mNGS and conventional methods increased the detection rate to 100%. Our data indicated that mNGS was valuable for the early detection of *Cryptococcus* reads in CSF.

Cryptococcal meningitis is an infectious disease of the CNS characterized by high morbidity and mortality[17]. With the increased number of immunocompromised patients, including those with AIDS, malignancies, and autoimmune diseases, cryptococcal meningitis has become a public health hazard[17]. However, cryptococcal meningitis manifests non-specific symptoms of fever, headache, vomiting, seizures, as well as focal neurological deficits, making its early diagnosis difficult[19]. However, early diagnosis and treatment contribute to a reduction in the morbidity and mortality rates of the disease. Despite being the gold standard, culturing is low-efficiency (0–40%) a and time consuming (taking 3–5 days). In our study, the positive rate of culturing fungi was only 63.4%, and the method can delay treatment due to the lengthy detection period. The sensitivity of India ink staining is 42–86% in cryptococcal meningitis cases (79.5% in our study), and can easily influenced by the competence of the operator, making it an unreliable method[11,20,21]. Therefore, CrAg, a fast and efficient diagnostic method has arisen. A comparative study[12] assessed the performance of the ME Panel in detecting cryptococcal infections, alongside CrAg tests and culture. The results revealed that the ME Panel demonstrated a positivity rate of 84.2% (32/38), whereas culture and CrAg tests yielded positivity rates of 73.7% (28/38) and 97.4% (37/38), respectively. These findings suggest that the ME Panel exhibits a high degree of concordance with culture, although its correlation with the CrAg test is slightly lower.

CrAg testing, with its superior sensitivity (over 90%), is a reliable method for diagnosing cryptococcal infections, though specific factors such as high antigen concentrations may influence its results in certain cases[22]. In our study, the initial CrAg test on the undiluted CSF sample from patient number 40 returned a negative result, while his serum sample tested positive. Additionally, a CSF fungal culture confirmed the growth of Cryptococcus neoformans, and mNGS detected Cryptococcus reads, both confirming the diagnosis of Cryptococcus neoformans meningitis. Upon further dilution of the CSF sample, repeated CrAg testing yielded a positive result with a high titer (> 1:2560). These findings suggest that the initial result may have been influenced by a post-zone effect due to an excessive concentration of CrAg in the CSF, rather than a true false negative. This highlights the importance of proper sample preparation and dilution to ensure accurate CrAg testing. Furthermore, when we performed CrAg testing on CSF samples from the 20 control patients, one patient (number 46) produced a positive result. The patient was admitted with cryptococcal pneumonia. During the treatment, this patient developed symptoms of meningitis. We performed a lumbar puncture, and subsequently, a variety of diagnostic methods were employed to identify different pathogenic microorganisms. The patient's CrAg testing was positive while India ink staining and culturing were both negative. mNGS was able to identify a purulent bacterium, *Nocardia* spp. without detecting *Cryptococcus* reads. To uncover whether the patient had a complicated infection of *Nocardia* spp. and *Cryptococcus*, we performed semi-quantitative CrAg testing on his CSF, which showed a titer of 1:2. And his serum CrAg titer is 1:5. Because of the low titer, we considered this to indicate no cryptococcal infection of his meninges. Subsequently, after antibacterial therapy alone, the patient's meningitis symptoms improved. However, one caveat may be added because the antibody used in CrAg testing was developed from a strain of *C. neoformans* s.l., false negatives may result from a poor affinity due to capsule-deficient *C. neoformans* s.l. or *C. gattii* s.l[23,24].

With high levels of replicability and objectivity, DNA sequencing has emerged as a new gold standard method for accurate species identification. A large-scale retrospective analysis with 511 CSF specimens from suspected infectious patients indicated that the sensitivity of mNGS was greater than cultures in detecting pathogens. Furthermore, mNGS was less commonly impacted by prior antibiotic exposures[25]. Additionally, mNGS offers a rapid and unbiased approach to the molecular diagnosis of infectious diseases. The turn around time for mNGS excluding transportation of specimen is 96 h, however, it is limited by the specific conditions and equipment required for its implementation. And this method allows for the identification of any pathogen without prior knowledge[26]. mNGS can be used not only for the diagnosis of cryptococcal meningitis, but also for the differential diagnosis such as the identification of *Mycobacterium tuberculosis*, *Nocardia*, *Herpes simplex virus*, etc. Meanwhile, mNGS can also identify mixed pathogens. In such instances, mNGS may serve as a powerful diagnostic tool in clinical practice. Our preliminary data suggested the potential utility of mNGS for detection of *Cryptococcus* at very low abundance. For example, patient number 20 who only produced 98 mNGS reads was diagnosed as negative based upon conventional CSF tests including fungal culturing and India ink staining. Furthermore, metagenomic next-generation sequencing (mNGS) analysis can not only identify the pathogen but also assess its antibiotic resistance genes. By sequencing the pathogen's genomic sequences in cerebrospinal fluid samples and comparing them to known antibiotic resistance gene databases, clinicians can obtain crucial information regarding the pathogen's resistance profile. This facilitates the selection of the most effective treatment strategy. This has been reported in the detection of Mycobacterium tuberculosis and fungal pathogens[27]. More significantly, for cryptococcal meningitis, mNGS can distinguish between *C. neoformans* and *C. gattii* which may benefit the diagnosis and management of cryptococcal meningitis, especially given that these two organisms require different courses of antifungal treatment[28]. Although the cost of mNGS is relatively high, its use can lead to substantial cost savings by reducing the duration of ineffective treatment and hospital stays for patients with cryptococcal meningitis, ultimately lowering the overall treatment expenses throughout the patient's care[29].

A recent report suggested the diagnostic sensitivity of mNGS (75%) was not superior to conventional methods (India ink staining, 83.33%, culturing, 83.33% and CrAg EIA, 100%)[30]. In our study, the sensitivity of

mNGS for the diagnosis of cryptococcal meningitis using CSF was 92.0%, similar to a prior study (93.5%) by Gan et al.[31]. The sensitivity still requires further improvement as an excellent diagnostic method for cryptococcal meningitis. For human samples, mNGS results comprise > 95% human reads, therefore, the removal of human DNA sequences is a major obstacle to the application of mNGS for diagnostics[32]. In addition, because *Cryptococcus* DNA is protected by a thick capsule, effective DNA extraction is another obstacle for detection of cryptococcal meningitis. Remaining barriers include contamination with external sources of nucleic acid, data analysis, method standardization, and interpretation challenges[33].

Our study reports a relatively large sample of patients in evaluation of the diagnostic performance of mNGS for cryptococcal meningitis that expands the limited body of literature on this topic, but it has several limitations. First, this was a retrospective study, which introduces the possibility of unrecognized biases and incomplete data collection including mNGS, CrAg, and CSF pressure measurements. Further investigation with larger samples is warranted to evaluate the diagnostic value of mNGS in cryptococcal meningitis. Second, the high cost of mNGS (3900 RMB3900RMB/550.29USD/416.52GBP) restricts its widespread use for detection in clinical practice. Additionally, as the peak time of pathogen occurrence may be different from the time first CSF is collected, repeat mNGS testing may improve sensitivity.

This study presents several limitations that warrant consideration. Firstly, as a retrospective analysis, it is inherently subject to challenges such as incomplete data and the potential for selection bias. The reliance on pre-existing records may result in gaps in information, which could impact the comprehensiveness of our findings and subsequently influence the outcome assessments. Secondly, the sample size in this study may be insufficient to ensure adequate statistical power, which limits the ability to generalize our findings to a broader population. Future studies with larger, more representative cohorts are essential to validate and extend the insights derived from our research.

Conclusions

This study evaluated the clinical features of patients with cryptococcal meningitis and compared the diagnostic performance of mNGS with conventional methods, including India ink staining, CrAg testing, and fungal culturing. Among the 65 patients recruited, 45 were diagnosed with cryptococcal meningitis based on pathogen detection in CSF, with the majority exhibiting neurological symptoms such as headache and elevated intracranial pressure. Notably, both mNGS and CrAg testing demonstrated high sensitivity, significantly outperforming the gold standard culturing method.

In conclusion, this study confirms that both the CrAg testing and mNGS have excellent sensitivity and specificity for diagnosing cryptococcal meningitis. Both methods are highly recommended for clinical practice due to their effective diagnostic performance.

CrAg testing is especially useful as a rapid and cost-effective screening tool, suitable for resource-limited settings and urgent cases, particularly in high-risk patients like those with advanced HIV. On the other hand, mNGS is better suited for complex cases with atypical symptoms or suspected co-infections, as it can identify a wider range of pathogens.

For optimal diagnostic accuracy, we suggest performing both CrAg testing and mNGS tests after the initial lumbar puncture in patients suspected of having cryptococcal meningitis. This approach ensures timely and comprehensive assessment for effective patient management.

Methods

Ethics statement and informed consent

This study was carried out in accordance with the recommendations, guidelines, and regulations using human specimens from the institutional review board of the First Affiliated Hospital of Fujian Medical University. All subjects or their legal representatives provided informed consent prior to inclusion. The retrospective-review protocol was approved by the institutional review board at the First Affiliated Hospital of Fujian Medical University.

Participants

A total of 65 patients who were admitted to the First Affiliated Hospital of Fujian Medical University between January 2020 and November 2022 were recruited (Figs. 2), 45 of whom were diagnosed with cryptococcal meningitis (meningoencephalitis). A definite cryptococcal meningitis diagnosis was made when at least one of India ink staining, fungal culture, mNGS, or CrAg test was positive from the CSF samples[17]. Raised ICP was defined as an opening pressure ≥ 25 cm of water[7]. Patients were managed in accordance with established clinical guidelines[7]. Twenty other patients with a definite diagnosis acted as controls, including 1 case of purulent meningitis, 17 cases of tuberculous meningitis, 1 case of herpes simplex virus encephalitis, and 1 case of anti-N-methyl-D-aspartate (NMDA) receptor (R) encephalitis. The purulent meningitis patient was detected via *Nocardia* DNA presence in his CSF by mNGS. Patients with tuberculous meningitis were positive on at least one of real-time PCR, mNGS, or Xpert MTB/RIF assays on their CSF samples[34]. Herpes simplex virus DNA was detected in the CSF of the positive patient via qPCR and mNGS[35]. Anti-NMDAR antibodies were detected in the CSF and serum of patients with anti-NMDAR encephalitis[36]. All cryptococcal meningitis and control patients underwent at least one type of diagnostic testing, either mNGS or one of several conventional tests, on their CSF samples collected during the first lumbar puncture after admission. Brain magnetic resonance imaging (MRI), and computed tomography (CT) scan were performed for each patient.

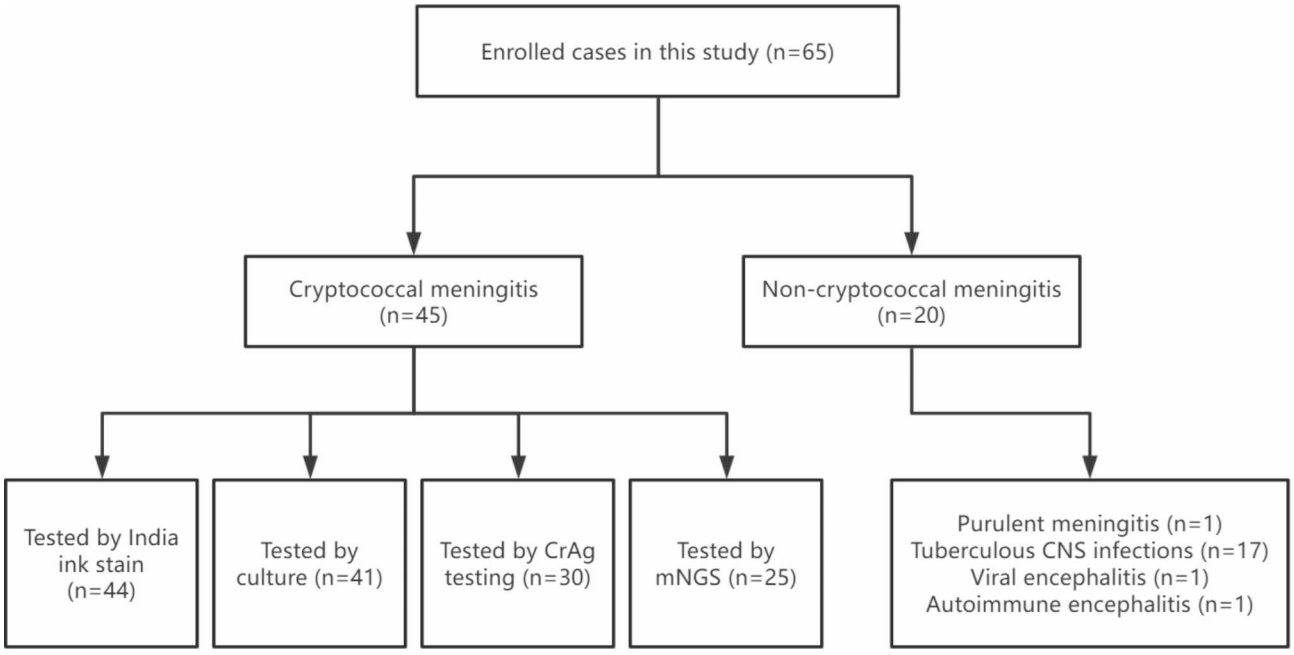

Fig. 2. Overview of patients enrolled, final diagnosis and number of patients tested by each method. A total of 65 patients were enrolled. Of these patients, 45 were diagnosed with CNS cryptococcal infection. As controls, 1 patient with purulent meningitis, 17 patients with tuberculous meningitis, 1 patient with viral encephalitis, and 1 patient with autoimmune encephalitis were enrolled.

India ink staining and fungal culture

India ink staining was performed to identify mucus substances and polysaccharide capsules of fungi[37]. The background of the slide was smeared with India ink (BA4042, Zhuhai Besso Biotechnology Co., Ltd) with the organisms themselves not colored, allowing the capsule of *Cryptococcus* to be visualized via negative staining. Fungal cultures began immediately after the completion of lumbar puncture, using BD BACTEC blood culture media bottles (Becton, Dickinson and Company, USA), and the fungal colonies were identified by mass spectrometry (Microflex LT/SH, Bruker Daltonics, Germany).

Cryptococcal antigen testing

Cryptococcal antigen was detected using a lateral flow assay (LFA). CSF from patients was tested using the CrAg LFA kit according to the manufacturer's instructions (IMMY, Norman, OK, USA). To avoid post-zone effects, all samples were diluted prior to testing. The CSF samples were diluted by mixing them in a 1:1 ratio with phosphate-buffered saline (PBS). The CrAg LFA test strip was then dipped into the sample and incubated for 10 min before reading the results. The test strip was colored upon cryptococcal antigen detection in the sample but remained white in the absence of the antigen. A quality control strip was always colored either for positive or negative samples.

Metagenomic next-generation sequencing

DNA extraction

Cell-free DNA was extracted from 2 mL of cerebrospinal fluid using the QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany). Cells in the cerebrospinal fluid were removed by centrifugation and the supernatant was collected for DNA extraction.

Library generation and sequencing

A DNA library was constructed according to the operating instructions of the QIAseq™ Ultralow Input Library Kit (QIAGEN, Hilden, Germany). Quality control of constructed library was performed using a Qubit 3.0 Of the two negative mNGS cases (Invitrogen, Q33216) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Qualified DNA libraries with diverse barcode tags were pooled and sequenced using the Nextseq 550 platform (Illumina, San Diego, CA, USA), with 75 bp single-end reads at a depth of approximately 20 million reads per library. After obtaining sequencing data, high quality reads were acquired by filtering out adapters, low quality, low-complexity, and short (<35 bp) reads. Next, human-derived sequences matching the human reference database (hg38) were removed by using SNAP software. The remaining reads were aligned to Microbial Genome Databases using Burrows–Wheeler Aligner software. This database contains a large collection of microbial genomes from NCBI representing more than 30,000 microorganisms, including 17,748 bacteria, 11,058 viruses, 1,134 fungi, and 308 parasites. The microbial composition of the samples was identified. The criteria for positive mNGS results were set as follows: (1) for bacteria other than TB, fungi other than *Cryptococcus* and parasites, sequencing coverage in the top 10 of all pathogens detected which is not detected in

the negative control (NTC); or sample/NTC with an RPM (reads per million mapping) ratio greater than 10. (2) For viruses, tuberculosis and cryptococci, at least 1 specific sequence detected, which is not detected in the NTC; or an RPM ratio of > 5 for samples/NTC.

Statistical analysis

Paired chi-square analysis was used to compare the sensitivity and specificity across mNGS and conventional tests. A p-value < 0.05 was considered significant. Not all patients had all four tests performed, therefore when conducting a paired chi-square analysis comparing the two tests we selected patients who had both tests performed.

Data availability

Sequence data filtering out the human genome of this study have been uploaded to the National Center for Biotechnology Information sequence read archives under project accession number PRJNA1192805 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA1192805).

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References

1. Coelho, C., Bocca, A. L. & Casadevall, A. The tools for virulence of Cryptococcus neoformans. *Adv. Appl. Microbiol.* **87**, 1–41. https://doi.org/10.1016/b978-0-12-800261-2.00001-3 (2014).
2. Stott, K. E. et al. Cryptococcal meningoencephalitis: Time for action. *Lancet. Infect. Dis.* **21** (9), e259–e271. https://doi.org/10.1016/s1473-3099(20)30771-4 (2021).
3. Vu, K. et al. Invasion of the central nervous system by Cryptococcus neoformans requires a secreted fungal metalloprotease. *mBio* **5**(3), e01101-14 (2014). https://doi.org/10.1128/mBio.01101-14
4. Tenforde, M. W. et al. Treatment for HIV-associated cryptococcal meningitis. *Cochrane Database Syst. Rev.* **25** (7), Cd005647. https://doi.org/10.1002/14651858.CD005647.pub3 (2018).
5. Lin, Y. Y., Shiau, S. & Fang, C. T. Risk factors for invasive Cryptococcus neoformans diseases: A case-control study. *PloS One*. **10** (3), e0119090. https://doi.org/10.1371/journal.pone.0119090 (2015).
6. Zhu, L. P. et al. Jun. Cryptococcal meningitis in non-HIV-infected patients in a Chinese tertiary care hospital, 1997–2007. *Med. Mycol.* **48**(4), 570–579 (2010). https://doi.org/10.3109/13693780903437876
7. Perfect, J. R. et al. Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the infectious diseases society of America. *Clin. Infect. Dis.* **1** (3), 291–322. https://doi.org/10.1086/649858 (2010).
8. Aye, C., Henderson, A., Yu, H. & Norton, R. Cryptococcosis-the impact of delay to diagnosis. *Clin. Microbiol. Infect.*. **22**(7), 632–635 (2016). https://doi.org/10.1016/j.cmi.2016.04.022
9. Moodley, A. et al. Early clinical and subclinical visual evoked potential and Humphrey's visual field defects in cryptococcal meningitis. *PloS One*. **7** (12), e52895. https://doi.org/10.1371/journal.pone.0052895 (2012).
10. Wilson, M. R. et al. Chronic meningitis investigated via metagenomic next-generation sequencing. *JAMA Neurol.* **75**(8), 947–955 (2018). https://doi.org/10.1001/jamaneurol.2018.0463
11. Rajasingham, R. et al. Cryptococcal meningitis diagnostics and screening in the era of point-of-care laboratory testing. *J. Clin. Microbiol.* **57** (1). https://doi.org/10.1128/jcm.01238-18 (2019).
12. Van, T. T., Kim, T. H. & Butler-Wu, S. M. Evaluation of the biofire filmarray meningitis/encephalitis assay for the detection of Cryptococcus neoformans/gattii. *Clin. Microbiol. Infect.* **26** (10), 1375–1379. https://doi.org/10.1016/j.cmi.2020.01.007 (2020).
13. Tansarli, G. S. & Chapin, K. C. Mar. Diagnostic test accuracy of the BioFire® FilmArray® meningitis/encephalitis panel: A systematic review and meta-analysis. *Clin. Microbiol. infect.* **26**(3), 281–290 (2020). https://doi.org/10.1016/j.cmi.2019.11.016
14. Xing, X. W. et al. Apparent performance of metagenomic next-generation sequencing in the diagnosis of cryptococcal meningitis: A descriptive study. *J. Med. Microbiol.* **68** (8), 1204–1210. https://doi.org/10.1099/jmm.0.000994 (2019).
15. Gu, W. et al. Rapid pathogen detection by metagenomic next-generation sequencing of infected body fluids. *Nat. Med.*. **27** (1), 115–124. https://doi.org/10.1038/s41591-020-1105-z (2021).
16. Brown, J. R., Bharucha, T. & Breuer, J. Encephalitis diagnosis using metagenomics: Application of next generation sequencing for undiagnosed cases. *J. Infect.* **76** (3), 225–240. https://doi.org/10.1016/j.jinf.2017.12.014 (2018).
17. Williamson, P. R. et al. Cryptococcal meningitis: Epidemiology, immunology, diagnosis and therapy. *Nat. Rev. Neurol.* **13** (1), 13–24. https://doi.org/10.1038/nrneurol.2016.167 (2017).
18. Marais, S. et al. Tuberculous meningitis: A uniform case definition for use in clinical research. *Lancet Infect. Dis.* **10** (11), 803–812. https://doi.org/10.1016/s1473-3099(10)70138-9 (2010).
19. Jin, K. et al. Nanopore sequencing of cerebrospinal fluid of three patients with cryptococcal meningitis. *Eur. J. Med. Res.* **3** (1), 1. https://doi.org/10.1186/s40001-021-00625-4 (2022).
20. Kwizera, R. et al. Acridine orange fluorescent microscopy is more sensitive than India ink light microscopy in the rapid detection of cryptococcosis among CrAg positive HIV patients. *PLoS One*. **12** (7), e0182108. https://doi.org/10.1371/journal.pone.0182108 (2017).
21. Boulware, D. R. et al. Multisite validation of cryptococcal antigen lateral flow assay and quantification by laser thermal contrast. *Emerg. Infect. Dis.* **20** (1), 45–53. https://doi.org/10.3201/eid2001.130906 (2014).
22. Aissaoui, N. et al. Investigation of CryptoPS LFA-positive sera in patients at risk of cryptococcosis. *Med. Mycol.* **3** (10). https://doi.org/10.1093/mmy/myac078 (2022).
23. Birkhead, M. et al. Cryptococcus neoformans: diagnostic dilemmas, Electron Microscopy and Capsular variants. *Trop. Med. Infect. Dis.* **4** (1). https://doi.org/10.3390/tropicalmed4010001 (2018).
24. McMullan, B. J., Sorrell, T. C. & Chen, S. C. Cryptococcus gattii infections: Contemporary aspects of epidemiology, clinical manifestations and management of infection. *Future Microbiol.* **8** (12), 1613–1631. https://doi.org/10.2217/fmb.13.123 (2013).
25. Miao, Q. et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin. Infect. Dis.* **13** (suppl_2), S231–s240. https://doi.org/10.1093/cid/ciy693 (2018).
26. Chiu, C. Y. & Miller, S. A. Clinical metagenomics. *Nat. Rev. Genet.* **20** (6), 341–355. https://doi.org/10.1038/s41576-019-0113-7 (2019).
27. Pham, D. et al. Molecular diagnostics for invasive fungal diseases: Current and future approaches. *J. Fungi (Basel Switzerland)* **26** (7). https://doi.org/10.3390/jof10070447 (2024).
28. Dou, H. T., Xu, Y. C., Wang, H. Z. & Li, T. S. Molecular epidemiology of Cryptococcus neoformans and Cryptococcus gattii in China between 2007 and 2013 using multilocus sequence typing and the DiversiLab system. *Eur. J. Clin. Microbiol. Infect. Dis.* **34** (4), 753–762. https://doi.org/10.1007/s10096-014-2289-2 (2014).

29. Tian, Y. et al. Economic impact of metagenomic next-generation sequencing versus traditional bacterial culture for postoperative central nervous system infections using a decision analysis mode: study protocol for a randomized controlled trial. *mSystems Dec.* **21** (6), e0058123. https://doi.org/10.1128/msystems.00581-23 (2023).
30. Xing, X-W. et al. Apparent performance of metagenomic next-generation sequencing in the diagnosis of cryptococcal meningitis: a descriptive study. *J. Med. Microbiol.* **68** (8), 1204–1210. https://doi.org/10.1099/jmm.0.000994 (2019).
31. Gan, Z. et al. Performance of metagenomic next-generation sequencing for the diagnosis of cryptococcal meningitis in HIV-negative patients. *Front. Cell. Infect. Microbiol.* **12**, 831959. https://doi.org/10.3389/fcimb.2022.831959 (2022).
32. Wang, H. et al. Metagenomic next-generation sequencing assists in the diagnosis of Cryptococcus pneumonia: Case series and literature review. *Front. Public. Health*. **10**. https://doi.org/10.3389/fpubh.2022.971511 (2022).
33. Hu, Z. et al. Metagenomic next-generation sequencing as a diagnostic tool for toxoplasmic encephalitis. *Ann. Clin. Microbiol. Antimicrob.* **17** (1). https://doi.org/10.1186/s12941-018-0298-1 (2018).
34. Wilkinson, R. J. et al. Tuberculous meningitis. *Nat. Reviews Neurol.* **13** (10), 581–598. https://doi.org/10.1038/nrneurol.2017.120 (2017).
35. Bradshaw, M. J. & Venkatesan, A. Herpes simplex Virus-1 encephalitis in adults: Pathophysiology, diagnosis, and management. *Neurotherapeutics: J. Am. Soc. Experimental Neurother. Jul.* **13** (3), 493–508. https://doi.org/10.1007/s13311-016-0433-7 (2016).
36. Peery, H. E. et al. Anti-NMDA receptor encephalitis. The disorder, the diagnosis and the immunobiology. *Autoimmun. Rev.* **11** (12), 863–872. https://doi.org/10.1016/j.autrev.2012.03.001 (2012).
37. Zerpa, R., Huicho, L. & Guillén, A. Modified India ink preparation for Cryptococcus neoformans in cerebrospinal fluid specimens. *J. Clin. Microbiol.* **34** (9), 2290–2291. https://doi.org/10.1128/jcm.34.9.2290-2291.1996 (1996).

Author contributions
Xiang-Ping Yao and Zai-Jie Jiang designed the study. Xiang-Ping Yao, Bi-Wei Lin, Wei-Qing Zhang, Qi-Chao Fan, Bi-Hui Yang and Zai-Jie Jiang were involved in the clinical management of these patients. Zai-Jie Jiang, Jian-Chen Hong and Xiang-Ping Yao wrote the article.

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Declarations

Competing interests
The authors declare no competing interests.

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