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RESEARCH

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Nanopore-targeted sequencing (NTS) for intracranial tuberculosis: a promising and reliable approach

Chen Yang[1†], Tianzhen Wang[2†], Yicheng Guo[1], Yi Zeng[1*] and Weiwei Gao[2*]

Abstract

Background The World Health Organization predicted 10.6 million new tuberculosis cases and 1.5 million deaths in 2022. Tuberculous meningitis, affecting 1% of active TB cases, is challenging to diagnose due to sudden onset, vague symptoms, and limited laboratory tests. Nanopore-targeted sequencing (NTS) is an emerging third-generation sequencing technology known for its sequencing capabilities. We compared its detection efficiency with Xpert, MTB culture, PCR, and AFB smear in cerebrospinal fluid samples to highlight the substantial potential of NTS in detecting intracranial tuberculosis.

Methods This study included 122 patients suspected of having intracranial tuberculosis at the Second Hospital of Nanjing in Jiangsu Province, China, between January 2021 and January 2024. The Univariate logistic regression and random forest regression identified risk factors and clinical markers. A chi-square test evaluated diagnostic accuracy for different image types of intracranial tuberculosis.

Results The research involved 100 patients with intracranial tuberculosis. Among them, 41 had tuberculous meningitis, 27 had cerebral parenchymal tuberculosis, and 32 had mixed intracranial tuberculosis. Besides, 22 patients were diagnosed with other brain conditions. In diagnosing intracranial tuberculosis, NTS demonstrated a sensitivity of 60.0% (95% CI: 49.7-69.5%) and a specificity of 95.5% (95% CI:75.1-99.8%), with an AUC value of 0.78 (95% CI: 0.71 to 0.84), whose overall performance was significantly better than other detection methods. There was no notable difference ($P > 0.05$) in diagnostic accuracy between NTS and the final diagnosis for intracranial tuberculosis patients with varying imaging types. Furthermore, patients who tested positive had a 31.500 (95% CI: 6.205-575.913) times higher risk of having intracranial tuberculosis compared to those with negative results.

Conclusion Due to its convenience, efficiency, quick turnaround time, and real-time sequencing analysis, NTS might become a promising and reliable method for providing microbiological diagnoses for patients with intracranial tuberculosis and for screening populations at risk.

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Keywords Nanopore-targeted sequencing(NTS), Tuberculous meningitis (TBM), Intracranial tuberculosis, Cerebrospinal fluid(CSF), Central nervous system(CNS)

Background

The Global Tuberculosis Report 2023 by the World Health Organization predicted that there would be 10.6 million new tuberculosis cases globally in 2022, with an expected 1.5 million deaths [1]. Tuberculous meningitis, a severe form of tuberculosis affecting the central nervous system, is caused by Mycobacterium tuberculosis(MTB) and accounts for about 1% of all active TB cases. It leads to significant morbidity and mortality, with approximately 250,000 new cases reported annually worldwide, one-third of which are in children. Tuberculous meningitis can result in severe disability and death in about half of patients [1–4]. However, diagnosing this condition is challenging due to its sudden onset, vague symptoms, and the limitations of traditional tests in detecting the bacteria in cerebrospinal fluid [5]. Currently, the diagnosis of tuberculous meningitis and even intracranial tuberculosis relies on a comprehensive assessment of the patient's clinical symptoms, laboratory findings, and imaging results. Anyway, it is still difficult to obtain a clear clinical diagnosis.

The Ziehl-Neelsen acid-fast Bacillus (AFB) smear technique in cerebrospinal fluid (CSF) is a commonly used and quick method for identifying tuberculous meningitis (TBM). However, the sensitivity of this method for diagnosing TBM is not considered adequate in clinical application due to the high concentration of MTB required in the CSF [6, 7]. The culture of CSF MTB is considered the gold standard for diagnosing tuberculous meningitis, with a low sensitivity range of 18.8–45.3% [8, 9]. However, the lengthy detection process is not ideal, making it challenging to diagnose TBM patients early [10]. Xpert MTB/RIF is a rapid and user-friendly molecular test recommended by the WHO for identifying MTB [11], providing results in just 2 h. While this method generally demonstrates high diagnostic effectiveness in typical tuberculosis patients, its efficacy drops notably when analyzing clinical samples with low bacterial loads [12, 13], such as cerebrospinal fluid specimens, achieving only a moderate sensitivity of 48.5-63.0% [14, 15], which hinders its broader application [16]. PCR is a promising molecular detection method that enables rapid diagnosis of Mycobacterium tuberculosis, and the sensitivity of detecting MTB in cerebrospinal fluid can vary from 32.4–57.4% [17, 18]. Nevertheless, challenges like nonspecific amplification, base mismatches, and the stringent requirements of primer design impede its complete clinical efficacy [19]. Therefore, developing rapid and accurate methods for diagnosing tuberculous meningitis from cerebrospinal fluid is crucial for improving patient outcomes [20].

Nanopore-targeted sequencing is an emerging third-generation sequencing technology known for its rapid sequencing capabilities, allowing for long-read sequencing of tens to hundreds of kilobases [21]. Besides, it can read and analyze sequencing data in real-time, making it effective for infectious disease monitoring and human genomics research [22, 23]. It is particularly adept at identifying GC repeat regions and base modification areas in MTB sequences, leading to its growing use in identifying this bacterium [24]. Currently, research is centered on the utilization of nanopore sequencing technology in cerebrospinal fluid samples to investigate the development of central nervous system infections [25]. However, there is a relative scarcity of studies on the clinical significance of nanopore-targeted sequencing in the diagnosis of TBM and even intracranial tuberculosis. Therefore, we aim to highlight the substantial diagnostic potential of nanopore-targeted sequencing for detecting intracranial tuberculosis by comparing its detection efficiency with Xpert, MTB culture, PCR, and AFB smear in cerebrospinal fluid samples.

Materials and methods

Study design and participants

We collected patients suspected of having intracranial tuberculosis at the Second Hospital of Nanjing in Jiangsu Province, China, between January 2021 and January 2024. To be eligible for the study, patients had to meet specific criteria: (1) having complete clinical data; (2) exhibiting symptoms of intracranial tuberculosis (such as fever, headache, vomiting, and consciousness disorder); (3) having only one brain disease; (4) being able to undergo lumbar puncture; (5) completing five specific tests simultaneously (NTS, Xpert, MTB culture, PCR, AFB smear). The study received approval from the Human Research Ethics and System Review Committee of the Second Hospital of Nanjing (ID: 2024-LS-ky026).

The diagnosis of intracranial tuberculosis in patients was determined based on their clinical features, microbiological and cerebrospinal fluid cytology test results, radiological findings, etc. The diagnostic criteria followed the most authoritative health industry standards in China: the Classification Standards for Tuberculosis (WS196-2017), the International Standard for tuberculous meningitis [26], the 2019 China Central Nervous System Tuberculosis Diagnosis and Treatment Guidelines [27], and expert consensus on intracranial tuberculosis imaging [28]. The study included 100 patients with

intracranial tuberculosis, comprising 41 with tuberculous meningitis, 27 with cerebral parenchymal tuberculosis, and 32 with mixed intracranial tuberculosis. Additionally, there were 22 patients with other brain diseases, including autoimmune encephalitis ($n=1$), viral encephalitis ($n=2$), intracranial tumors ($n=3$), cryptococcal encephalitis ($n=3$), and suppurative encephalitis ($n=13$). The study's workflow is illustrated in Fig. 1.

Specimen collection and informed consent

The day after being admitted to the hospital with suspected intracranial tuberculosis, a lumbar puncture was performed on the patient following the exclusion of any contraindications and without the use of medications. A 15 ml sample of fresh cerebrospinal fluid was collected and placed directly into a sterile test tube, sealed, and stored in a refrigerator at -80 °C. This sample was then used for various tests including routine examinations (appearance, cell count, etc.), biochemical tests (glucose, protein, chloride, etc.), NTS, Xpert, MTB culture, PCR, and AFB smear. The aforementioned tests utilized 2 ml of cerebrospinal fluid for detection purposes, while the remaining 1 ml was retained as a backup to prevent any potential bias resulting from improper handling. It was ensured that the collection of clinical specimens respects the privacy and interests of patients, and informed consent was obtained from both patients and their families before collecting samples, with all patients signing consent forms.

Acid-fast bacilli (AFB) smear and mycobacterium tuberculosis (MTB) culture

The 2022 update of the Practical Manual on Tuberculosis Laboratory Strengthening by the World Health Organization (WHO) outlines the meticulous adherence to laboratory protocols for tuberculosis testing [29]. Acid-fast staining microscopy includes the preparation of a smear from a patient's sample, staining it with a dye (such as Auramine O), heating the smear, and subsequently examining it under a microscope to detect the presence of mycobacteria. 1. Utilizing the MGIT 960 detection system, a mixture of 1 ml of cerebrospinal fluid (CSF) and 0.8 ml of Middlebrook 7H9 Broth Base for MGIT culture was prepared in the MGIT tube. The tube was then placed in a 37 °C BACTEC MGIT 960 system (Becton Dickinson; Franklin Lakes, NJ, USA) for incubation. To address the challenge of prolonged detection cycles in the cultivation process, we have decided to extend the cultivation period of Mycobacterium tuberculosis (MTB) negative MGIT tubes by an additional eight weeks to improve the detection rate of MTB. This strategy is particularly crucial for patients suspected of having intracranial tuberculosis, as they may exhibit lower levels of Mycobacterium tuberculosis. By increasing the culture

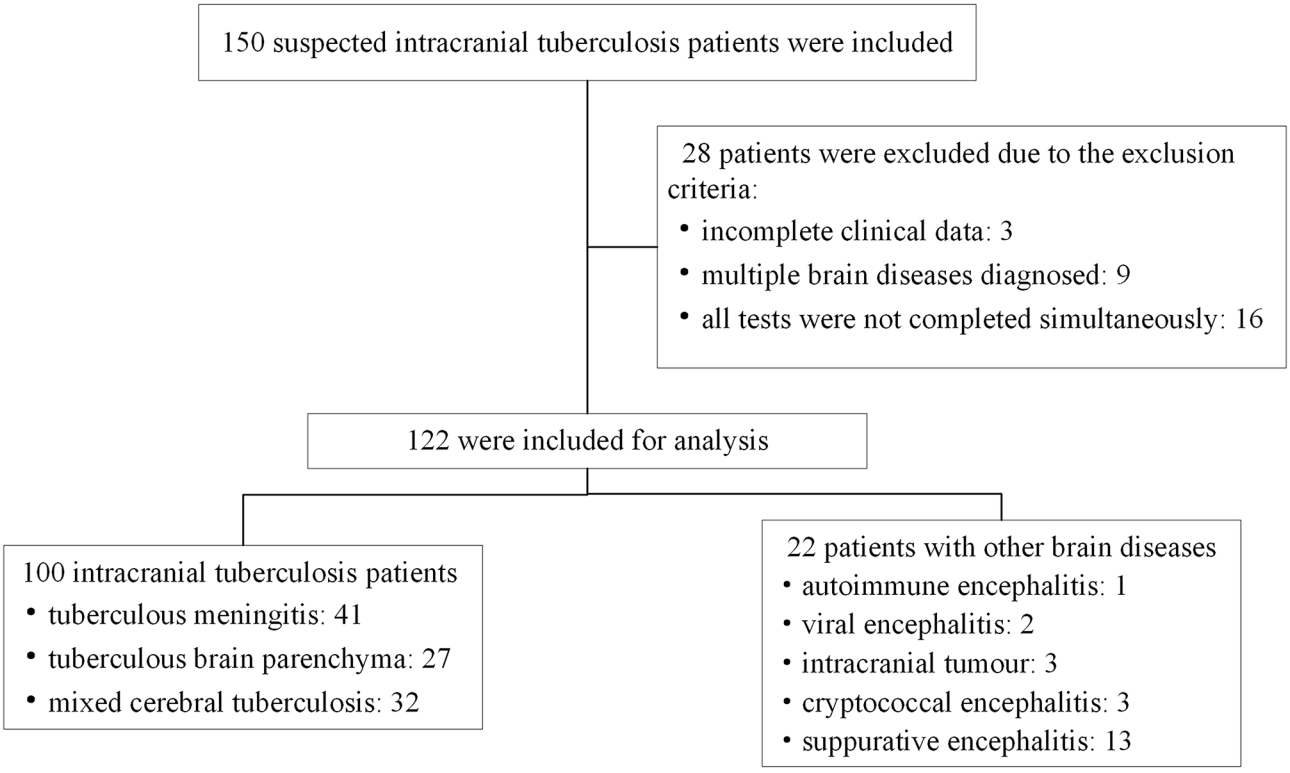

Fig. 1 Workflow of the categorization of patients involved in the study

duration, we could enhance the likelihood of detecting MTB, resulting in more accurate diagnostic outcomes for patients.

Polymerase chain reaction (PCR)

Before conducting PCR, we assessed the quality of the DNA extracted from cerebrospinal fluid by utilizing a spectrophotometer to measure absorbance at 260/280 nm. A ratio of A260/A280 ranging from 1.8 to 2.0 was deemed indicative of high-quality DNA. Furthermore, we employed gel electrophoresis to verify the integrity of the DNA prior to proceeding with PCR. The PCR amplification was performed following a standard protocol that included specific cycles of denaturation, annealing, and extension. The temperature and duration for each phase were optimized according to the design of the primers and the properties of the target gene. We included positive controls with known DNA samples containing the MPB64 gene to validate the PCR reaction and to ensure that the assay was functioning correctly. Meanwhile, each PCR run included negative controls with no template DNA to monitor for contamination during the extraction and amplification processes. After PCR, the amplified products were analyzed by gel electrophoresis using a 2% agarose gel. A DNA ladder was run in parallel to estimate the size of the amplified fragments. The gel was stained with ethidium bromide and visualized under UV light to confirm the presence of the expected 240 bp fragment. Each sample was processed in duplicate to ensure consistency and to identify any potential errors or inconsistencies in the results.

Xpert MTB/RIF

Xpert MTB/RIF (Cepheid, Sunnyvale, United States) was used to detect MTB DNA in specimens. The cerebrospinal fluid (CSF) was mixed with the sample treatment reagent provided in the Xpert MTB/RIF assay kit (Cepheid, Sunnyvale, United States) at a ratio of 1: 2. This means that for 1 mL of CSF, 2 mL of the sample treatment reagent was added. The mixture was then allowed to liquefy for 15 min. Subsequently, the mixture was transferred into the GeneXpert cartridge using the sterile dropper included in the kit and inserted into the GeneXpert machine. The automated GeneXpert system displayed the results within 2 h.

Nanopore targeted sequencing (NTS)

The DNA extraction and purification were performed following the instructions provided by the QIAamp DNA microbiome kit (QIAGEN, Canada). The quantification of the extracted genomic DNA was done using the Qubit fluorometer 4.0 (Thermo Fisher Scientific, MA, USA). Subsequently, the extracted DNA underwent PCR amplification, which was carried out on the ABI 2720 Thermal Cycler (Applied Biosystems, USA). Following the guidelines of the Native Barcoding Expansion 1–12 (Oxford Nanopore Technologies, UK), the purified product was labeled with a barcode. After that, a 1 μL sample was taken and the concentration of the extracted DNA was measured using the Invitrogen Qubit 4 fluorometer (Thermo Fisher Scientific, MA, USA). The pooling libraries were constructed using the DNA Ligation Kit SQK-LSK110 (Oxford Nanopore Technologies, UK) as per the manufacturer's protocol, and the sequencing was conducted on a Nanopore instrument (GridION X5, Oxford Nanopore Technologies, UK). All sequencing procedures were carried out by Zhejiang ShengTing Biotech. Co., Ltd. (Hangzhou, China).

Statistical analysis

In this research, categorical variables were depicted using frequency and ratio, while continuous variables were represented by median and interquartile range. The study calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the five detection methods by analyzing TP, FP, FN, and TN values. Combined with ROC curves and the Venn diagram, the effectiveness of NTS and other detection methods in diagnosing intracranial tuberculosis was compared. Univariate logistic regression and random forest regression were employed to study the factors contributing to the risk of intracranial tuberculosis and to identify key clinical markers for predicting the occurrence of intracranial tuberculosis. The diagnostic accuracy of the five detection methods for different types of intracranial tuberculosis images was evaluated using a chi-square test. The utility of NTS in analyzing low-load clinical specimens, such as cerebrospinal fluid samples from patients with intracranial tuberculosis, was demonstrated through locally enlarged scatter plots. All statistical analyses were performed on R version 4.4.1.

Results

Characteristics of the participants included in the study

In this research, out of the 100 individuals diagnosed with intracranial tuberculosis, 48 were male (48.0%) with a median age of 50.0 [IQR: 30.0, 64.0]. Among the clinical symptoms observed, fever was present in 65 patients (65.0%) with intracranial tuberculosis and 19 patients (86.4%) with other brain conditions. Headache was reported by 49 individuals (49.9%) and 17 individuals (77.3%), while dizziness was experienced by 30 patients (30.0%) and 17 patients (77.3%), respectively. A total of 12 (12.0%) and 2 (9.1%) patients had consciousness disorders, respectively. In terms of cerebrospinal fluid cytology, the median number of cerebrospinal fluid cells in patients with intracranial tuberculosis was 4.0*10^6 [IQR: 1.0, 27.0], significantly lower than the median

number in patients with other brain diseases, which was 138.5*10^6 [IQR: 10.0, 232.8] ($p<0.001$). The median cerebrospinal fluid pressure in patients with intracranial tuberculosis was 14.0 mmol/L [IQR: 10.0, 17.0], lower than the median pressure in patients with other brain diseases, which was 23.0 [IQR: 16.0, 34.0] ($p<0.001$). The median CSF protein content in patients with intracranial tuberculosis was 422.5 mg/L [IQR: 193.0, 816.5], higher than the median in patients with other brain diseases, which was 179.0 mg/L [IQR: 100.8, 273.3] ($p=0.004$). The study identified 41 patients with meningeal tuberculosis, 27 with parenchymal tuberculosis, and 32 with mixed intracranial tuberculosis based on imaging types. Table 1 summarizes the demographic and clinical characteristics of the patients in the study.

Comparisons of diagnostic performance of NTS and the other tests for intracranial tuberculosis

Follow the recognized diagnostic criteria for tuberculosis in China, including clinical information, lab test outcomes, and imaging results, to assess if the research participant has intracranial tuberculosis. Use this assessment as a standard to evaluate the diagnostic performance of all testing approaches. In diagnosing intracranial tuberculosis, the sensitivity of NTS, Xpert, MTB culture, PCR, and AFB smear were 60.0 (95%CI:

Table 1 Demographic and clinical characteristics of the included patients

Characteristic	Overall($n=122$)	Intracranial tuberculosis ($n=100$)	Other brain diseases ($n=22$)	*P*-value
Age	52.0[31.0,64.8]	50.0[30.0,64.0]	56.0[48.0,65.0]	0.190
Gender				0.275
Male	62(50.8)	48(48.0)	14(63.6)	
Female	60(49.2)	52(52.0)	8(36.4)	
Fever				0.088
Yes	84(68.9)	65(65.0)	19(86.4)	
No	38(31.1)	35(35.0)	3(13.6)	
Headache				0.030
Yes	66(54.1)	49(49.0)	17(77.3)	
No	56(45.9)	51(51.0)	5(22.7)	
Dizziness				< 0.001
Yes	47(38.5)	30(30.0)	17(77.3)	
No	75(61.5)	70(70.0)	5(22.7)	
Nausea and vomiting				0.001
Yes	30(24.6)	18(18.0)	12(54.5)	
No	92(75.4)	82(82.0)	10(45.5)	
Consciousness disorder				0.986
Yes	14(11.5)	12(12.0)	2(9.1)	
No	108(88.5)	88(88.0)	20(90.9)	
Hypomnesia				0.550
Yes	10(8.2)	7(7.0)	3(13.6)	
No	112(91.8)	93(93.0)	19(86.4)	
Cerebral infarction				0.056
Yes	34(27.9)	32(32.0)	2(9.1)	
No	88(72.1)	68(68.0)	20(90.9)	
Cerebral hemorrhage				0.950
Yes	3(2.5)	3(3.0)	0(0.0)	
No	119(97.5)	97(97.0)	22(100.0)	
Image type				0.080
Meningeal	56(45.9)	41(41.0)	15(68.2)	
Brain parenchymal	31(25.4)	27(27.0)	4(18.2)	
Mixed	35(28.7)	32(32.0)	3(13.6)	
CSF pressure(/cmH_2O)	15.0[10.0,18.4]	14.0[10.0,17.0]	23.0[16.0,34.0]	< 0.001
Cell count (/*10^6)	6.0[1.0,60.0]	4.0[1.0,27.0]	138.5[10.0,232.8]	< 0.001
Protein(mg/L)	342.0[156.0,758.3]	422.5[193.0,816.5]	179.0[100.8,273.3]	0.004
Glucose(mmol/L)	3.5[2.8,4.1]	3.5[2.9,4.1]	3.5[2.7,3.7]	0.478
Chloride(mmol/L)	123.0[116.0,126.0]	123.0[116.0,126.0]	121.0[113.3,125.5]	0.209
ADA(U/L)	1.0[0.0,4.0]	1.0[0.0,4.0]	4.0[0.0,5.0]	0.160

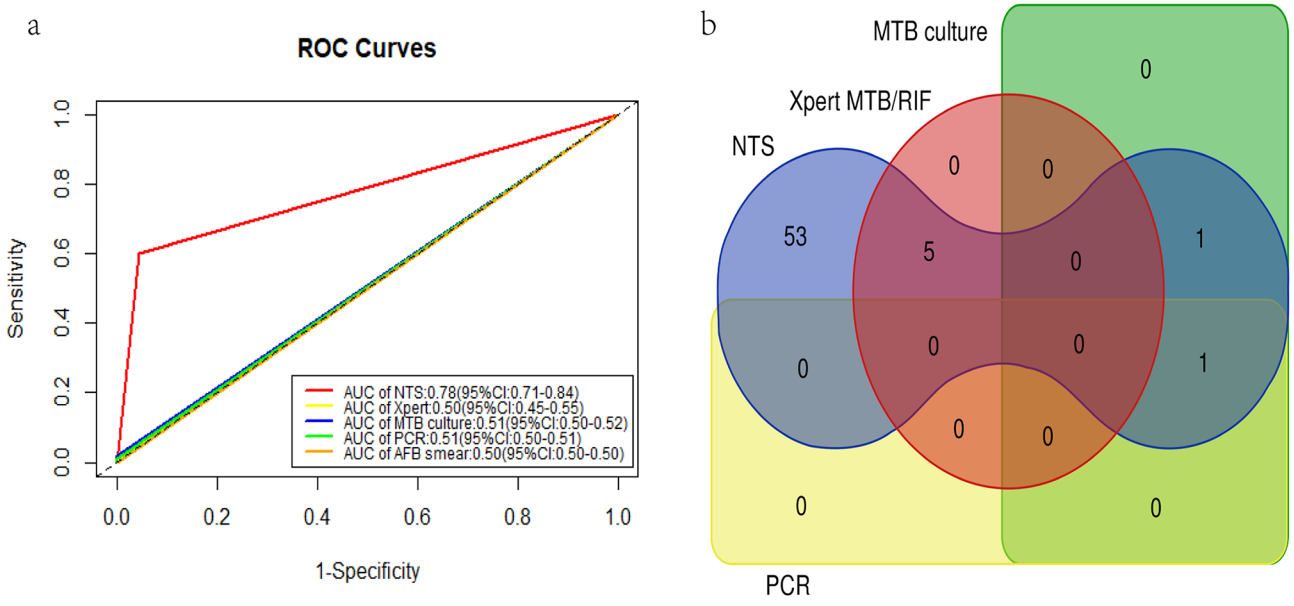

Fig. 2 **a** ROC curves of the five tests for intracranial tuberculosis. **b** Venn diagram of positive tests for intracranial tuberculosis patients

Table 2 Diagnostic efficiency of the five tests for intracranial tuberculosis

Test	Sensitivity(%,95%CI)	Specificity(%,95%CI)	PPV(%,95%CI)	NPV(%,95%CI)	AUC(95%CI)
NTS	60.0(49.7–69.5)	95.5(75.1–99.8)	98.4(90.0-99.9)	34.4 (23.0-47.8)	0.78(0.71–0.84)
Xpert	5.0(1.9–11.8)	95.5(75.1–99.8)	83.3(36.5–99.1)	18.1(11.8–26.6)	0.50(0.45–0.55)
MTB culture	2.0(0.3–7.7)	100.0(81.5–100.0)	100.0(19.8–100.0)	18.3(12.1–26.7)	0.51(0.50–0.52)
PCR	1.0(0.1–6.2)	100.0(81.5–100.0)	100.0(5.5–100)	18.2(12.0-26.5)	0.51(0.50–0.51)
AFB smear	0.0(0.0-4.6)	100.0(81.5–100.0)	/	18.0(11.9–26.3)	0.50(0.50–0.50)

PPV: positive predictive value; NPV: negative predictive value; AUC: area under the curve; MTB: Mycobacterium tuberculosis; AFB: acid-fast bacilli

49.7–69.5), 5.0 (95%CI: 1.9–11.8), 2.0 (95%CI: 0.3–7.7), 1.0(95%CI:0.1–6.2) and 0.0(95%CI:0.0-4.6), respectively. The specificity were 95.5 (95%CI: 75.1–99.8), 95.5 (95%CI: 75.1–99.8), 100.0 (95%CI: 81.5–100.0), 100.0 (95%CI: 81.5–100.0), and 100.0 (95%CI: 81.5–100.0), respectively. The AUC values were 0.78 (95%CI: 0.71–0.84), 0.50 (95%CI: 0.45–0.55), 0.51 (95%CI: 0.50–0.52), 0.51 (95%CI: 0.50–0.51), and 0.50 (95%CI: 0.50–0.51), respectively. Figure 2.a displays the ROC curves of each detection method. Table 2 summarizes the effectiveness of all tests in detecting intracranial tuberculosis. When considering the positive results of all tests for patients with intracranial tuberculosis, NTS could independently detect 53 patients with intracranial tuberculosis, which was 0 among other tests. Additionally, NTS shared 5 positive results with Xpert, 1 positive result with MTB culture, and 1 positive result with PCR and MTB culture. These findings are illustrated in Fig. 2.b.

Comparisons of effectiveness of NTS and other clinical indicators for diagnosing intracranial tuberculosis

Figure 3.a displays the outcomes of NTS and various clinical markers in predicting the risk of intracranial tuberculosis using univariate logistic regression. The categorical variables are binary, with 1 denoting presence or positive and 0 indicating absence or negative. The findings revealed that positive NTS results, elevated cerebrospinal fluid protein levels, and cerebral infarction are statistically significant risk factors for intracranial tuberculosis (OR>1, $p<0.05$). In particular, patients with positive NTS results have a significantly higher risk (OR=31.500, 95%CI: 6.205-575.913) of developing intracranial tuberculosis compared to those with negative results. Figure 3.b illustrates the contribution of NTS and other clinical features in predicting intracranial tuberculosis occurrence through random forest regression. The importance scores of different features are ranked, with cerebrospinal fluid pressure, NTS results, and dizziness symptoms being the top contributors to the prediction. In summary, NTS plays a crucial role in the supplementary diagnosis of intracranial tuberculosis.

Diagnostic accuracy of the five tests for different imaging types of brain diseases

In this study, 100 cases of intracranial tuberculosis were categorized based on the image type. These included 41 cases of meningeal tuberculosis, 27 cases of brain parenchymal tuberculosis, and 32 cases of mixed tuberculosis.

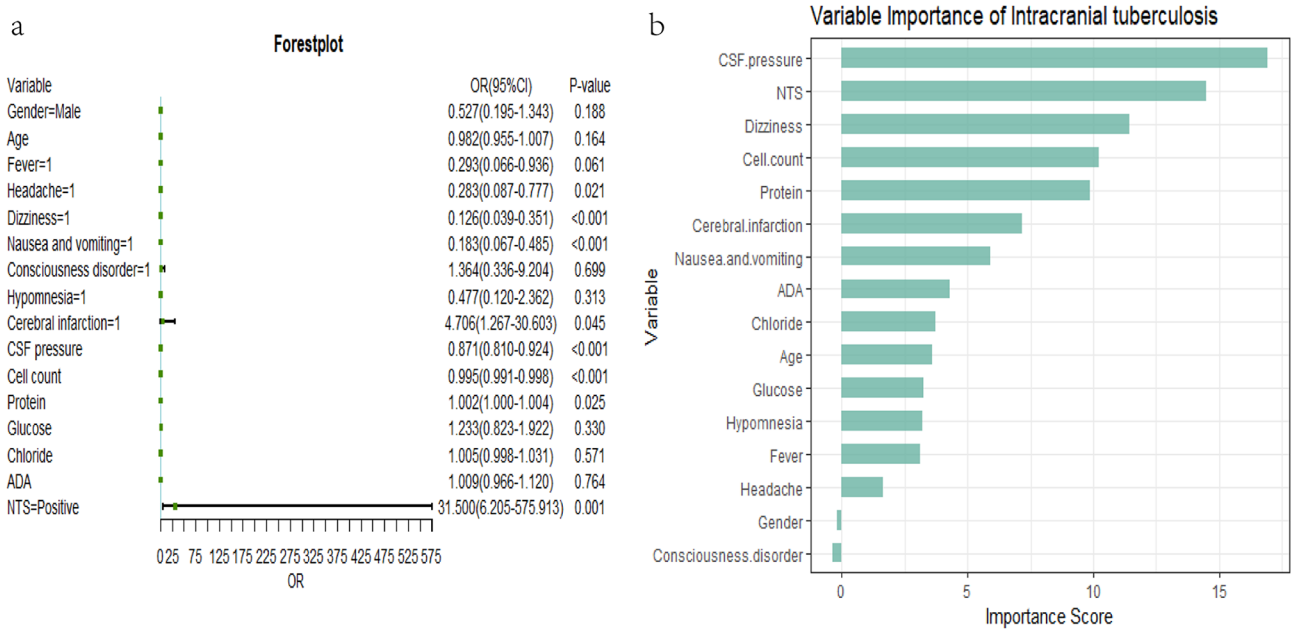

Fig. 3 **a** Forest plot of different variables for predicting the risk of intracranial tuberculosis. **b** Diagram of variable importance in predicting the occurrence of intracranial tuberculosis

Table 3 Diagnostic accuracy of the five tests for diagnosing intracranial tuberculosis and other brain diseases

Test	Meningeal		Brain parenchymal		Mixed		Overall	
	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value	χ^2	*P*-value
NTS	0.408	0.523	0.724	0.439	0.299	0.675	1.375	0.241
Xpert MTB/RIF	20.287	< 0.001	15.242	0.002	2.933	0.143	37.937	< 0.001
MTB culture	20.287	< 0.001	11.215	0.007	17.371	0.002	48.207	< 0.001
PCR	20.287	< 0.001	15.242	0.002	17.371	0.002	51.549	< 0.001
AFB smear	23.077	< 0.001	15.242	0.002	17.371	0.002	55.217	< 0.001

Among the 22 cases of other brain diseases, 15 were found in the meninges, 4 in the brain parenchyma, and 3 in both areas. The study found that there was no significant variance ($P>0.05$) in the diagnostic accuracy between NTS and final diagnosis for brain diseases solely affecting the meninges or brain parenchyma (Table 3). Similarly, there was no statistically significant difference ($P>0.05$) in diagnostic accuracy between NTS and Xpert for brain diseases affecting both the meninges and brain parenchyma compared to the final diagnosis (Table 3). Overall, our study concluded that there was no significant distinction ($P=0.241$) in the accuracy of diagnosing intracranial tuberculosis and other brain diseases using NTS (Table 3).

Tuberculosis sequence numbers NTS detected in different imaging types of intracranial tuberculosis

NTS can directly identify the nucleotide sequence of Mycobacterium tuberculosis in clinical samples like cerebrospinal fluid. In this study, Fig. 4 displays the tuberculosis sequence detected by NTS in patients with intracranial tuberculosis, showcasing the Mycobacterium tuberculosis content in cerebrospinal fluid samples. It revealed that 56 out of 60 cases (93.3%) of intracranial tuberculosis patients had a tuberculosis sequence number below 40 in their cerebrospinal fluid samples, suggesting a low bacterial load. The Kruskal-Wallis test indicated no significant difference in CSF bacterial load among patients with different types of intracranial tuberculosis imaging ($P=0.24$), demonstrating the suitability of NTS for all imaging scenarios.

Discussion

Intracranial tuberculosis is a severe infectious disease caused by Mycobacterium tuberculosis (MTB) affecting the central nervous system, leading to high rates of mortality and disability [30]. The clinical diagnosis of this condition is challenging due to vague symptoms, unusual imaging results, the limitations of cerebrospinal fluid (CSF) analysis for differential diagnosis, and the low sensitivity of microbiological tests on CSF, which can result in delayed, missed, or incorrect diagnoses [31–33]. Early detection is crucial for effective treatment to significantly

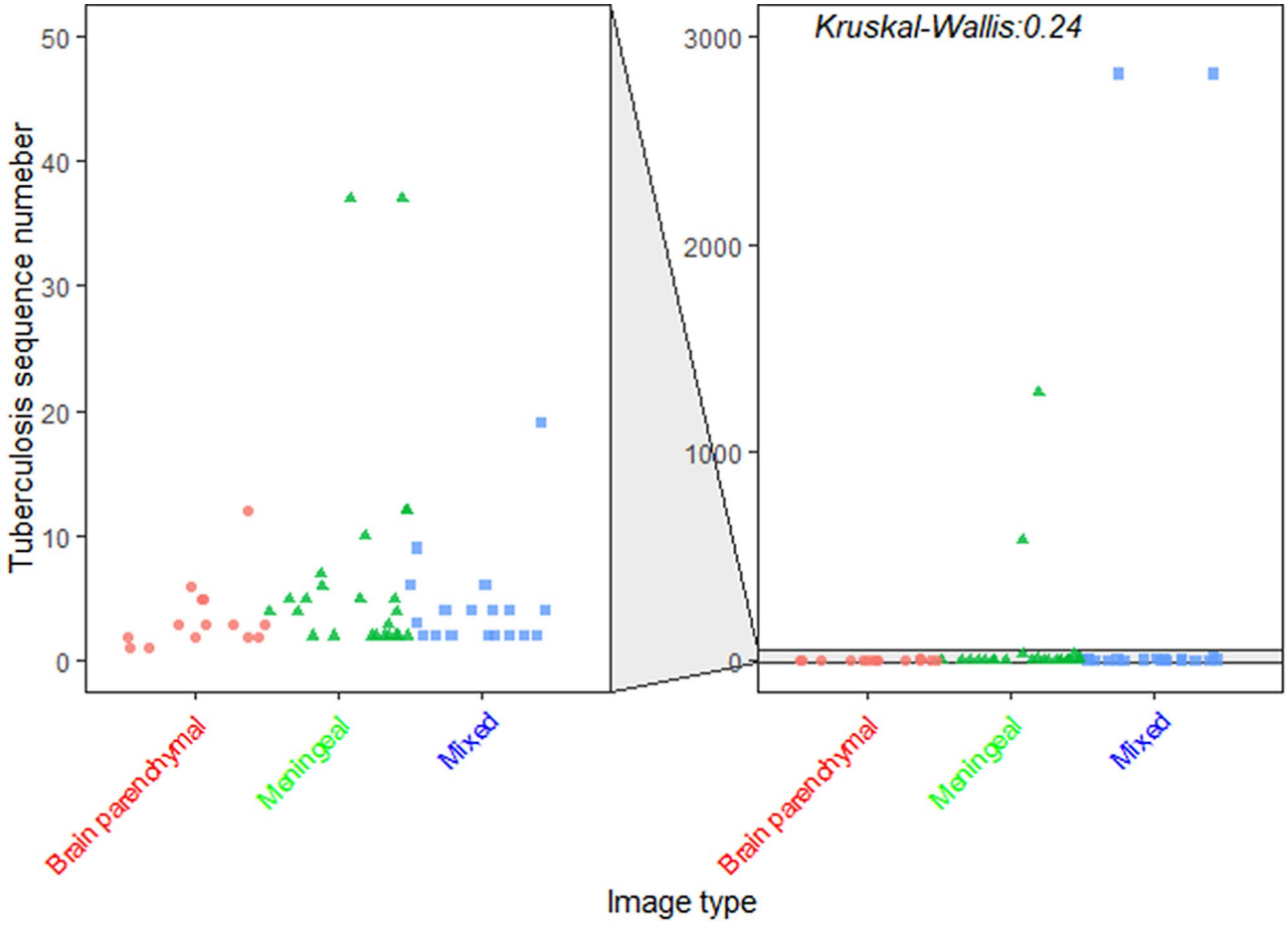

Fig. 4 Scatter plot of tuberculosis sequence number NTS detected in intracranial tuberculosis patients

lower mortality and disability rates, particularly in cases of tuberculous meningitis.

Unfortunately, more than half of intracranial tuberculosis cases lack microbiological diagnostic support due to the paucibacillary nature of the disease [34]. The key issue is to enhance the detection efficiency of CSF samples from affected patients. Conventional detection methods are no longer appropriate for clinical settings. The low concentration of MTB in CSF, the limited volume of CSF available for testing, and the impact of sample preparation and interpretation methods contribute to traditional diagnostic methods' low positive detection rates [35]. In this study, CSF acid-fast bacilli (AFB) smear yielded no positive results, and the sensitivity of MTB culture was only 2.0% (95% CI: 0.3-7.7%), similar to previous research [36]. Surprisingly, the two most commonly used molecular tests, Xpert and PCR, performed poorly in our study, with sensitivities of 5.0% (95% CI: 1.9-11.8%) and 1.0% (95% CI: 0.1-6.2%), respectively. As nanopore-targeted sequencing (NTS) can directly quantify tuberculosis sequences in clinical samples, we found that 93.3% (56/60) of CSF samples from 60 patients with intracranial tuberculosis contained fewer than 40 MTB copies, and 83.3% (50/60) had fewer than 10 copies (Fig. 4). Thus, we believe that a possible reason for the abnormal sensitivity of Xpert and PCR is that the content of MTB in CSF samples in this study is too low. Nevertheless, NTS demonstrated a relatively high detection sensitivity of 60.0% (95% CI: 49.7-69.5%), largely due to its technological advantages. NTS can enrich the specific nucleic acid sequences of MTB in clinical specimens through targeted capture technology, providing more data for detection and enabling early and rapid tuberculosis diagnosis, particularly in cases with low bacterial loads [37]. This also clarifies why NTS is more effective than other tests in diagnosing MTB in cerebrospinal fluid. Our research indicated that, in contrast to other detection methods, which had limited diagnostic performance (AUC values of 0.50–0.51), NTS achieved an AUC value of 0.78 (95% CI: 0.71–0.84), indicating strong diagnostic value for patients with intracranial tuberculosis (Fig. 2.a). Furthermore, among the 60 patients with intracranial tuberculosis identified by NTS, 53 were identified solely by NTS, while 5 were shared with Xpert, 1 with MTB culture, and 1 with both MTB culture and PCR. Notably, no other tests were able to identify any patients with intracranial

tuberculosis on their own (Fig. 2.b). These demonstrated that NTS had significant advantages in detecting clinical specimens with low bacterial counts. Moreover, NTS offered a relatively quick turnaround time, averaging approximately 10 h. This was consistent with the 8–14 h turnaround time reported by Fu Y et al. [38] for detecting infectious specimens using the same method, and it was significantly shorter than the 2–8 weeks required for MTB culture detection, which is considered the gold standard. To improve the positive detection rate of MTB through culture, we allowed for an additional 8 weeks of reculturing; however, the sensitivity of NTS remained considerably higher than that of the culture method (60.0%>>2.0%). While techniques such as AFB smear, GeneXpert MTB/RIF, and PCR had slightly quicker turnaround times than nanopore-targeted sequencing, they did not achieve sufficient positive detection rates. We assert that NTS uniquely provides a balance of rapid turnaround and high positive detection rates, making it an effective and reliable tool for the early and targeted treatment of tuberculosis.

NTS not only offers microbiological diagnoses for patients with intracranial tuberculosis but also shows significant promise for quickly screening at-risk populations. One key advantage is that the detection process can be performed using a single MinION nanopore sequencer, which is portable and requires minimal investment [23]. Another benefit is that the MinION features a flow cell with 512 channels, each containing 4 nanopores, allowing for a total of 2048 nanopores for DNA or RNA sequencing. This multiplexing capability speeds up the turnaround time and greatly lowers the sequencing cost per sample [39]. Additionally, NTS operates on the principle of detecting changes in current as DNA/RNA passes through the nanopore, enabling the determination of nucleotide sequences and modifications in real time [40]. Given these advantages, NTS is deemed highly suitable for the real-time monitoring of infectious diseases and the rapid screening of high-risk groups [41–43]. Our research indicated that positive NST test outcomes, symptoms such as dizziness or cerebral infarction, along with increased protein levels or pressure in cerebrospinal fluid, may suggest the presence of intracranial tuberculosis (Fig. 3). Among them, the most significant was the positive NTS test result. As depicted in Fig. 3.a, patients who tested positive for NTS had a 31.5 (95% CI: 6.205-575.913, $p=0.001$) times higher risk of developing intracranial tuberculosis compared to those who tested negative. Frankly, there was one false positive in the NTS test, which involved a brain tumor being incorrectly identified as intracranial tuberculosis, possibly due to accidental contamination during testing. On the other hand, the clinical practicability of other factors is limited. While tuberculous meningitis often results in dizziness or cerebral infarction, it has been reported that miliary tuberculosis can lead to blood system abnormalities during dissemination, which may also cause cerebral infarction or dizziness [44], suggesting that these clinical symptoms lack specificity. Besides, nearly all infectious diseases affecting the central nervous system can elevate cerebrospinal fluid protein levels and cerebrospinal fluid pressure. It has been documented that five adult patients with autoimmune glial fibrillary acidic protein (GFAP) astrocytopathy were incorrectly diagnosed with TBM because their cerebrospinal fluid showed high protein levels and elevated pressure, leading to the ineffectiveness of anti-tuberculosis treatment [45]. In conclusion, we maintained that positive NTS results could serve as a significant indicator of the risk of intracranial tuberculosis and play an important role in the screening of high-risk groups.

Our study has certain limitations. It is a single-center retrospective study with a small sample size, leading to patient selection bias. Moreover, we did not observe any drug resistance results in cerebrospinal fluid samples from patients with intracranial tuberculosis using NTS, which is unexpected considering the significance of drug resistance in TB diagnosis and treatment. Nevertheless, literature is scarce on detecting drug resistance in intracranial tuberculosis patients using NTS. Future research may require multi-center and large-sample studies to validate the use of NTS in identifying drug resistance in patients with intracranial tuberculosis.

Conclusion

Due to its convenience, efficiency, quick turnaround time, real-time data analysis, and other benefits, nanopore-targeted sequencing is a promising method for providing microbiological diagnoses for patients with intracranial tuberculosis and for screening populations at risk.

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Author contributions

Chen Yang and Tianzhen Wang were responsible for the conceptual design of the experiments and the validation of the methods, while Yicheng Guo handled the organization and evaluation of the research data. Chen Yang conducted the statistical analysis, and along with Tianzhen Wang, compiled the final results and prepared the research draft. Yi Zeng and Weiwei Gao oversaw project management and experimental supervision. All authors have reviewed and given their approval for the final manuscript.

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Data availability
The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval
The study received approval from the Human Research Ethics and System Review Committee of the Second Hospital of Nanjing (ID: 2024-LS-ky026).

Consent to participate
Informed consent was obtained from all the participants.

Consent for publication
Informed consent was obtained from all the participants in this study as well as the co-authors.

Competing interests
The authors declare no competing interests.

Conflict of interest
All authors have no conflict of interest to declare.

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