Journal of Microbiology, Immunology and Infection 58 (2025) 340–346

Contents lists available at ScienceDirect

Journal of Microbiology, Immunology and Infection

journal homepage: www.e-jmii.com

Clinical value of Metagenomic Next- and Third-Generation Sequencing applied in ultrasound-guided puncture biopsy for diagnosing lymph node tuberculosis

Ting Lin [a,1], Yuehui Yu [b,1], Jialei Luo [a], Xinyi Yan [b], Yuxuan Qiu [e], Jiahui Tong [e], Ying Wang [e], Xiangyun Huang [c], Dan Li [c], Ying Zhang [d], Gaoyi Yang [e,*]

[a] The Fourth School of Clinical Medicine, Zhejiang Chinese Medical University, Hangzhou First People's Hospital, PR China
[b] Hangzhou Normal University, PR China
[c] Key Laboratory of Digital Technology in Medical Diagnostics of Zhejiang Province, Dian Diagnostics Group Co, Ltd., Hangzhou, Zhejiang Province, PR China
[d] Department of Ultrasonography, Affiliated Hangzhou Red Cross Hospital, PR China
[e] Affiliated Hangzhou First People's Hospital, School of Medicine, Westlake University, PR China

ARTICLE INFO

Keywords:
Cervical lymph node
Tuberculosis
Metagenomic next-generation sequencing
Metagenomic third-generation sequencing
Ultrasound-guided puncture biopsy

ABSTRACT

Objectives: The aim of this study was to assess the clinical utility of metagenomic next-generation sequencing (mNGS) and metagenomic third-generation sequencing (mTGS) in diagnosing cervical lymph node tuberculosis through analysis of lymph node tissue.
Methods: This study recruited 101 patients with suspected cervical lymph node tuberculosis and took samples under ultrasound guidance. Parallel culture, X-pert, mNGS and mTGS workflows to each sample were performed. Taking clinical diagnosis as the gold standard, We comparatively compared diagnosis performance of the four methods.
Results: Clinically, 76 cases were diagnosed as lymph node tuberculosis and 20 cases were non-lymph node tuberculosis. Compared with clinical diagnosis, the sensitivities of mNGS and mTGS were 89.47 % and 82.89 %, respectively, significantly higher than those of culture and X-pert which were 43.28 % and 68.42 %, respectively ($P < 0.05$). The specificity of mNGS and X-pert reached 100 %, while those of culture and mTGS were 93.75 % and 95 %, respectively. And mNGS alone identified 7 species of bacteria, 3 species of viruses, and 5 species of fungi, and identified more mixed infections. Particularly, besides *Mycobacterium tuberculosis* detection, mNGS may be superior to mTGS for the detection of fungi. Pathogen identification of mNGS and mTGS is less affected by previous anti-tuberculosis drug usage.
Conclusion: mNGS and mTGS play a crucial role in the rapid diagnosis and accurate treatment on Cervical lymph node tuberculosis.

1. Introduction

The Global Tuberculosis Report 2023 indicated that *Mycobacterium tuberculosis* (*M.tuberculosis*, TB) maintains the second leading cause of infectious disease-causing death globally.[1] It is highlighted that over 10 million people became infected with TB every year. Additionally, the number of TB patients has increased from 10.3 million in 2021 to 10.6 million in 2022, further exacerbating the financial payments.[1] Tuberculosis, commonly known as pulmonary tuberculosis, may also affect other bodily organs, which is referred to as extrapulmonary tuberculosis (EPTB). It is noteworthy that EPTB account for about 15 % of all individuals infected with TB.[1] Lymph node tuberculosis, a type of EPTB, which has the highest incidence rate, usually poses a significant challenge to accurately diagnose based on the presentation of clinical symptoms and imaging findings alone,[2] which can result in the delayed diagnosis and poor prognosis.

* Corresponding author.
E-mail addresses: Linting1831769450@163.com (T. Lin), yuyuehui99@126.com (Y. Yu), luojialei991224@163.com (J. Luo), yanxinyi0518@163.com (X. Yan), wangying0329y@163.com (Y. Wang), 21817056@zju.edu.cn (X. Huang), lidan15@dazd.cn (D. Li), zhangying5201@163.com (Y. Zhang), yanggaoyi@hospital.westlake.edu.cn (G. Yang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jmii.2024.12.011
Received 28 August 2024; Received in revised form 4 December 2024; Accepted 31 December 2024
Available online 4 January 2025

Several tools have been applied to diagnosis for TB infection in clinical. Microbial culture remains the gold standard method. However, the cultivation cycle of *M. tuberculosis* is too long (about 2–6 weeks in liquid media), and the result of cultivation highly dependent upon the skill and experience of expert, which increase the possibility of delay and misdiagnosis.[3] The X-pert MTB is a culture-independent diagnostic tool that can provide results for a specific microorganism within 2 h. Despite the reported increased diagnostic efficiency of X-pert in cervical tuberculous lymphadenitis by *Meng* et al.,[4] the sensitivity of X-pert is not entirely satisfactory. When the load of *M. tuberculosis* in the sample is low, it may be lower than the threshold that X-pert can effectively detect, thus leading to a false negative result. Therefore, there is an urgent need for a rapid and accurate method for the differential diagnosis of lymph node tuberculosis.

In recent years, rapid advancement has been observed in high-throughput sequencing technology, particularly in terms of its culture-independent and unbiased nature.[5,6] Such technologies, including metagenomic next-generation sequencing (mNGS) and metagenomic third-generation sequencing (mTGS), are now being increasingly utilized in clinical settings for a wide range of applications.[7–9] Reviewing literature, mNGS can reliably identify emerging or causative microbes in unexplained infectious diseases, assisting in clinical precision diagnosis and successful treatment.[10–13] In addition, mTGS with long-sequencing on Nanopore platform represented a suitable alternative and/or complement in a clinical setting.[14] It was a feasibility tool for pathogen detection and species identification of *M. tuberculosis*.[15] At present, there have been many studies reporting its clinical application in tuberculosis. However, there is still a lack of large-scale clinical validation in cervical lymph node tuberculosis. Besides, there is no study comparing the performance differences between mNGS and mTGS in cervical lymph node tuberculosis.

In our study, 101 patients suspected of cervical lymph node tuberculosis were enrolled. By comparing with traditional pathogen diagnostic methods including culture and X-pert tests, we clarify the clinical significance of mNGS and mTGS in the diagnosis of cervical lymph node tuberculosis, and explore their differences in diagnostic performance, in order to lay the foundation for the application of metagenomics in this field.

2. Method

2.1. Participants and study design

Lymph node tissue specimens used in the study were obtained from 101 patients with suspected cervical lymph node tuberculosis through ultrasound-guided puncture, which were enrolled from Hangzhou Red Cross Hospital between December 2022 to November 2023. Samples that met the inclusion criteria were detected for pathogens through four methods, including *Mycobacterium tuberculosis* culture, X-pert, metagenomic next-generation sequencing (mNGS) and metagenomic third-generation sequencing (mTGS). Inclusion criteria were as below: 1) Clinicians highly suspected that the patient had lymph node tuberculosis based on clinical characteristics and imaging; 2) lymph node tissue. Exclusion criteria: 1) Patients with incomplete clinical information; 2) Patients with insufficient sample volume; 3) Patients with co-infection with HIV; 4) Patients who did not agree to participate.

Combining the criteria of Expert consensus on the diagnosis and treatment of superficial lymph node tuberculosis and medical records, 76 patients was classified as cervical lymph node tuberculosis and 20 were lymph node enlargement or tumor-related diseases. Remaining five patients were identified as uncertain diseases. The diagnosis criteria was followed as below: 1) Any patient with symptoms and signs of superficial lymph node tuberculosis and a positive microbiological diagnosis of *Mycobacterium tuberculosis* can be diagnosed as lymph node tuberculosis; 2) If the ultrasound examination of the patient is consistent with the relevant manifestations of lymph node tuberculosis, accompanied by confirmation through immunological examination and effective diagnostic anti-tuberculosis treatment, superficial lymph node tuberculosis can be clinically diagnosed. This study was approved by the Medical Ethics Committee of Hangzhou Red Cross Hospital and was conducted according to the principles of the Helsinki Declaration.

2.2. Ultrasound-guided puncture sampling and specimen fixation

The patients were required to be in a supine position with full exposure of the focal side of the neck, and the all examinations were performed by a sonographer with >10 years of experience. Grayscale imaging, color doppler imaging and contrast-enhanced ultrasound were performed for each patient. The contrast agent used for CEUS was SonoVue. Firstly, the target lymph nodes were located through a two-dimensional ultrasound scan of the neck, and the blood flow of the lymph nodes was observed. Then a nurse injected contrast agent (2.4 mL) intravenously, and the probe was fixed to observe the filling of the focal lymph nodes with contrast agent. Dynamic CEUS images were maintained for 2 min. According to the CEUS image, the puncture site was selected to fill the lesion tissue with contrast agent. The patient's local skin was routinely disinfected 3 times, then local stratified anesthesia was administered with 2 % lidocaine hydrochloride, and needle biopsy was performed when ready. The biopsy was performed by a qualified interventional sonographer and the biopsy sampling equipment (Bard, Covington, KY, USA) was 18G (the diameter of the cutting needle was 1.2 mm; In real-time monitoring of 2D ultrasound, the length of the sampling notch should be 1 or 2 cm depending on the size of the lesion). Major blood vessels and tissues should be avoided during puncture biopsy. A coarse needle biopsy was performed on the enhanced area. From each case, 2–4 tissue samples are taken.

The tissues were placed in specimen cups and submitted respectively to the laboratory for fully automated *Mycobacterium tuberculosis* culture, X-pert detection, mNGS, and mTGS.

2.3. Mycobacterium tuberculosis culture assay

Mycobacterium tuberculosis was cultured by utilizing the BACTEC MGIT960 liquid culture reagents and system. The specimens collected were subjected to decontamination with 1 % N-acetyl-L-cysteine (NALC)/sodium hydroxide (NaOH) followed by centrifugation. Tissues were homogenized mechanically and then floated in sterile saline solution. Thereafter, the resultant suspension was adopted for mycobacterium culture via the BACTEC MGIT960 mycobacterium liquid culture system.

2.4. X-pert assay

Took 1 ml of the ground tissue fluid and put it into the pretreatment tube. Added 2 mL of the pretreatment solution to it. Shook it for 20 s and then let it stand for 15 min. Aspirated 2 mL of the treated sample solution and injected it into the reaction box through the sample injection port. Placed the reaction box in the automated detection module for detection. The system could automatically read the detection results after 2 h.

2.5. mNGS and mTGS assays

2.5.1. Sample processing

Tissue samples were subjected to a pre-treatment protocol to facilitate subsequent DNA extraction and sequencing. These frozen tissue samples were sectioned into fragments and soaked in a PBS solution for thorough grinding. Then effective host DNA removal was performed as below, 100 μl of the tissue slurry was mixed evenly with 60 μL of a treatment solution (PNA:PNB = 0.5:20) and 440 μL of PTD, the resulting mixture was incubated at 37 °C at 1000 rpm for 20 min. Subsequently, 6 μL of PPT was added into the mixture. After thorough broken, the

mixture was centrifuged and the supernatant was aspirated into a 1.5 mL sterile centrifuge tube.

2.5.2. DNA extraction, mNGS test and bioinformatic analysis

Basically, according to the manufacturer's instructions, the supernatant from tissue pretreatment was used to isolate DNA using magnetic beads method (Genskey Co., Ltd, China). After calibrating DNA concentration, DNA library was constructed by DNA enzyme digestion, end-repair, barcode ligation, library purification, and PCR amplification according to the manufacturer's instructions ((NEBNext Ultra II DNA Library Prep Kit for Illumina), New England Biolabs Inc.).[16] The quality of DNA library was assessed using the Qubit dsDNA High-Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, USA). Subsequently, the qualified DNA library was sequenced on MGISEQ-200RS sequencing platform (MGI, China), using MGISEQ-200RS high-through sequencing regents kit (FCL SE50).[17] Among the raw data, low-quality reads, adaptor contamination, duplicate reads, and those shorter than 50bp were all filtered through fastp software. Host DNA sequences that aligned to human genome reference sequence hg38 were removed.[18] The remaining data were multisequence aligned and standardized at 20 million (M) for each sample. Microorganism identification of filtered data aligned to in-house genomic database (Dian diagnostics pathogenic microorganism genome database).

2.5.3. DNA extraction, mTGS test and bioinformatic analysis

DNA extraction, library construction and sequencing were conducted in accordance with previously reported methods.[19] Briefly, DNA extraction was performed according to the manufacture's instructions ((TIANamp Bacteria DNA Kit), TIANGEN, China). Qubit reagent (ThermoFisher Scientific, Invitrogen, USA) was utilized to accurately calibrate DNA concentration. Following fragmentation, barcode ligation, library purification, and PCR amplification according to the manufacture's instructions (SQK-RPB004 Rapid PCR Barcoding Kit (Nanopore), Oxford Nanopore Technologies, UK), the quality of DNA library was assessed using Qubit detection. Eventually, the qualified DNA library was sequenced based on GridION platform (Oxford Nanopore Technologies, UK) as previously described (MGI Tech Co., Ltd., Shenzhen, China).[19,20]

Raw data was sequenced through MinION sequencer (Oxford Nanopore Technologies, UK), a real-time identification and the fastq files was generated using MinKnow software. MinKnow software was applied to filter out low-quality sequences, subsequently eliminating host DNA that aligned to human genome reference sequence Hg38 using Minimap2 Software. Filtered data was multisequence aligned and standard as 20 million (M) for each sample. Microorganism identification of the filtered data was carried out at the National Center for Biotechnology Information, which was a nonredundant nucleic acid database (https://www.ncbi.nlm.nih.gov/).[19]

2.5.4. Pathogen identification criteria

As reported previously, the reported criteria of pathogens were established following the type of clinical samples and detected microorganisms.[19–21] Considering the original blast results would contain redundant micro-ecosystem microorganisms, the reported criteria of pathogens were created within lymph node. For the clinical core pathogens, such as tuberculosis (TB), whose sequences no less than one would be considered as positive. For the clinical-relevant microbes, whose sequences no less than three would be reported. Others that never isolated from clinical infections would be reported with more than twenty sequences.

2.5.5. Statistical analysis

Continuous variables were expressed as median and interquartile range, and continuous variables that did not conform to a normal distribution were tested for significance by Mann-Whitney test. Binary variables were expressed as counts, and the results of sensitivity and specificity were expressed as 95 % confidence intervals, and the significance of sensitivity was tested by McNemar test. The significance of the positive rate among different detection was tested by *chi*-square test. All data were statistically analyzed using SPSS 27.0. $P < 0.05$ is considered as significant statistically.

3. Results

3.1. Clinical characteristics of patients

A total of 101 patients suspected of lymph node tuberculosis were enrolled in this study. Among them, approximately 72 % (73/101) of the individuals were female. The median age of the patients was 39 years old. A smaller proportion of patients received drug treatment before consultation, including 18 (17.8 %) cases of anti-tuberculosis therapy and 3 (3.0 %) cases of antibacterial usage. Among the underlying diseases, pulmonary tuberculosis is the most common, which accounts for about one-third of the proportion (Table .1).

3.2. The diagnostic ability of four methodologies for M. tuberculosis

Among these individuals, 76 patients were classified as lymph node tuberculosis, lymph node enlargement or tumor-related diseases were finally diagnosed in 20 cases, remaining 5 patients failed to obtained a certain clinical diagnosis (Fig. 1).

We analyzed the performance in the *Mycobacterium tuberculosis* detection of four methodologies (Culture, X-pert, mNGS and mTGS) (Fig. 2). In terms of seventy-six cases with lymph node tuberculosis, compared to culture and X-pert tests, the rate of *Mycobacterium tuberculosis* detection through mNGS (89.47 %) was higher, which was similar to that through mTGS (82.89 %). In terms of twenty individual with lymph node enlargement or tumor-related diseases, X-pert and mNGS was negative for *Mycobacterium tuberculosis* detection, while culture and mTGS were positive for only one cases, respectively. Though *Mycobacterium tuberculosis* was detected in more cases through mNGS and mTGS, the consistency of *Mycobacterium tuberculosis* detection through different methodologies for each case was further compared.

The concordance between mNGS assay and traditional tests for *Mycobacterium tuberculosis* detection is displayed in Fig. 3A. Seventeen cases were both positive for culture, X-pert, and mNGS assays. besides, and 5 cases were both negative for culture, X-pert, and mNGS assays. For the 10 cases with no overlapping in the *Mycobacterium tuberculosis* detection, including only mNGS positive (8 cases), only X-pert positive (2 cases).

The concordance between mTGS assay and traditional tests for *Mycobacterium tuberculosis* detection is displayed in Fig. 3B. Seventeen cases were both positive for culture, X-pert, and mTGS assays. besides, and 8 cases were both negative for culture, X-pert, and mTGS assays. For

Table 1
Clinical characteristics of 101 patients suspected of lymph node tuberculosis.

Characteristics	value
Female, n (%)	73 (72.3 %)
Age, median (IQR)	39 (28–54.5)
Anti-tuberculosis therapy, n (%)	18 (17.8 %)
Antibacterial therapy, n (%)	3 (3.0 %)
Underlying disease, n (%)	
Hypertension	6 (5.9 %)
Diabetes	4 (4.0 %)
Pulmonary tuberculosis	30 (29.7 %)
Tumor	2 (2.0 %)
Chronic liver disease	5 (5.0 %)
Chronic kidney disease	1 (1.0 %)
Digestive system diseases	1 (1.0 %)
Thyroid diseases	2 (2.0 %)

IQR, interquartile range.

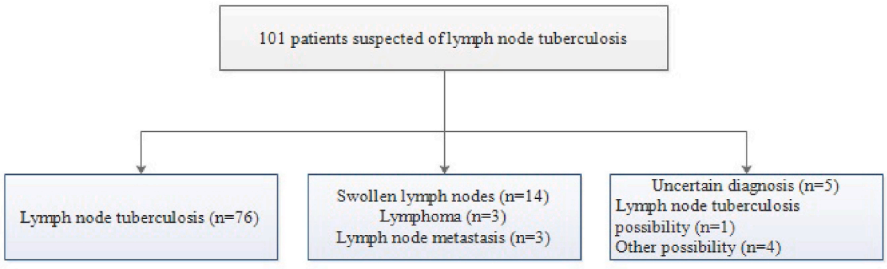

Fig. 1. Clinical diagnosis.

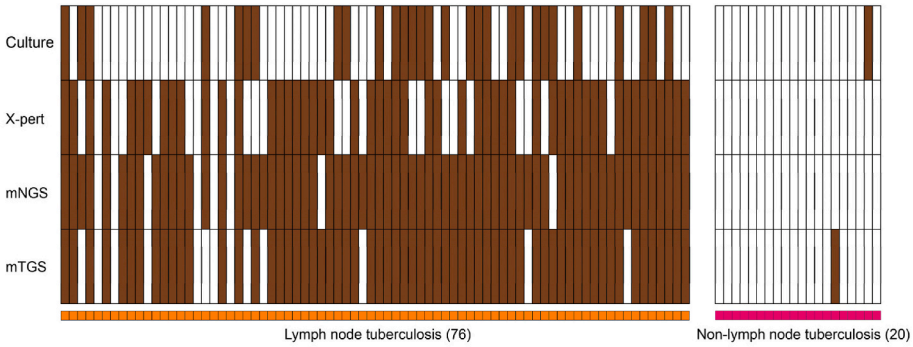

Fig. 2. Heatmap of the diagnostic ability of different methodologies for *Mycobacterium tuberculosis*.

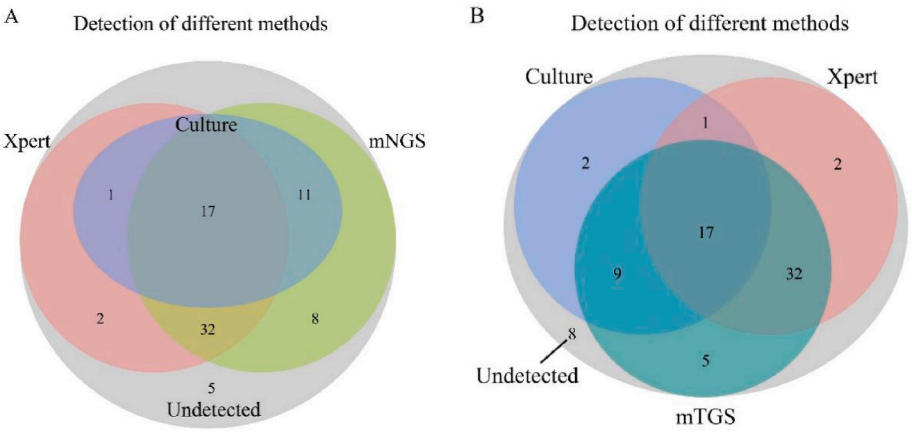

Fig. 3. Venn diagram of Detection of different methods in patients with lymph node tuberculosis.

the 9 cases with no overlapping in the *Mycobacterium tuberculosis* detection, including only mTGS positive (5 cases), only X-pert positive (2 cases), only culture positive (2 cases). These results indicated that the performance of mNGS and mTGS was similar. Their false negative results may be due to sampling. The amount of pus in the lesion of most lymph node tuberculosis patients is difficult to support a one-time high-throughput sequencing. Ultrasound-guided needle aspiration biopsy becomes a feasible approach. In this case, whether the puncture sample is close enough to the lesion directly determines the positive or negative result of high-throughput sequencing.

Taking clinical diagnosis as gold standard, the sensitivity and specificity of each methodology were analyzed (Table .2 and Table .3). The sensitivity of X-pert was significantly higher than that of culture (68.42 % vs 43.28 %) (p p < 0.001). The specificities of X-pert and mNGS reached 100 %, while those of culture and mTGS were 93.75 % and 95 %, respectively. Due to the high sensitivity demonstrated by both X-pert and high-throughput sequencing, the combination of these two methods

Table 2
Diagnostic performance of the Culture, X-pert, mNGS and mTGS compared to clinical diagnosis.

Detection method	Sensitivity (95%CI, n/N)	Specificity (95%CI, n/N)
Culture	43.28 % (0.314–0.559, 29/67)	93.75 % (0.677–0.997, 15/16)
X-pert	68.42 % (0.566–0.783, 52/76)	100.00 % (0.791–1.000, 19/19)
mNGS	89.47 % (0.798–0.950, 68/76)	100.00 % (0.800–1.000, 20/20)
mTGS	82.89 % (0.722–0.902, 63/76)	95.00 % (0.731–0.997, 19/20)
X-pert + mNGS	93.42 % (0.847–0.976, 71/76)	100.0 % (0.800–1.000, 20/20)
X-pert + mTGS	86.84 % (0.767–0.932, 66/76)	95.00 % (0.731–0.997, 19/20)

Table 3
P value of the McNemar test on the sensitivity between different methodologies.

method	Culture	X-pert	mNGS	mTGS
Culture				
X-pert	0.017			
mNGS	<0.001	<0.001		
mTGS	<0.001	0.008	0.344	

effectively improved the identification ability of *Mycobacterium tuberculosis*. The sensitivity of X-pert + mNGS reached 93.42 %. Therefore, the diagnostic ability of mNGS and mTGS for *M. tuberculosis* was comparable and significantly better than traditional pathogenic detection methods.

3.3. Overview of pathogenic detection through mNGS and tNGS

Since our hospital is a tuberculosis specialist hospital, traditional pathogenic methods mainly target *M. tuberculosis*. Therefore, besides *M. tuberculosis*, we also compared pathogen identification between mNGS and mTGS (Fig. 4). Among the 101 enrolled patients, the positive rates in pathogen detection of mNGS and mTGS were 89.11 % (90/101) and 81.19 % (82/101), respectively. Among them, 9 kinds of bacteria, 5 kinds of viruses, and 5 kinds of fungus, mainly including *M. tuberculosis* (69/90), *K. pneumoniae* (4/90), *Human herpesvirus 4* (30/90), and *Torque teno virus* (13/90). While, mTGS detected 6 kinds of bacteria and 3 kinds of viruses, mainly including *M. tuberculosis* (66/82), *Human herpesvirus 4* (20/82), *Human herpesvirus 7* (5/82). The mNGS alone detected 7 kinds of bacteria, including 1 strain of *M. chelonae*, 1 strain of *M. gadium*, 1 strain of *M. avium*, 2 strains of *M. abscessus*, 1 strain of *M. kansasii*, 1 strain of *Enterococcus faecalis*, and 1 strain of *Bacteroides vulgatus* were only detected by mNGS And 3 kinds of bacteria, including 2 strain of *Stenotrophomonas maltophilia*, 1 strain of *Legionella maceachernii* and 1 strain of *Haemophilus influenzae* were only detected by mTGS. As for viral, mNGS alone detected 14 strains of *Torque teno virus*, 3 strains of *Human herpesvirus 6*, and 1 strain of *Human herpesvirus 5*, while mTGS only detected 1 strain of *Human parvovirus B19* alone. All fungus were detected by mNGS. In terms of infection type, single infection was mainly bacterial infection (mNGS, 44/90; mTGS, 57/82). However, the mNGS detected more coinfections, including bacterial-viral coinfection (29 cases *vs* 13 cases), bacterial-fungal coinfection (1 case *vs* 0), and bacterial-viral-fungal coinfection (1 case *vs* 0). The mNGS detected more potential pathogenic microorganisms.

3.4. Effect of anti-tuberculosis therapy on high-throughput sequencing results

We screened the enrolled patients who underwent all four tests and divided them into two groups according to whether or not they used anti-tuberculosis drugs before the test, anti-tuberculosis therapy group (n = 15) and no anti-tuberculosis therapy group (n = 72). We analyzed that the impact of anti-tuberculosis drugs on the results of the detection and the number of high-throughput sequencing reads (Table .4). Obviously, the use of anti-tuberculosis drugs significantly reduced the positive rate of culture (40.28 % vs 6.67 %, $X^2 = 6.207$, $p = 0.013$). However, the use of drugs increased the positive rate of high-throughput sequencing, but it was not statistically significant (86.67 % vs 68.06 %, $X^2 = 1.289$, $p = 0.256$; 80.00 % vs 65.28 %, $X^2 = 0.651$, $p = 0.420$). Meanwhile, anti-tuberculosis treatment did not have a significant impact on the reads of high-throughput sequencing ($p = 0.815$; $p = 0.578$). The increase in the positive rates of X-pert and high-throughput sequencing may be due to patient referrals and rechecks, therefore, the pathogen has not been completely eliminated.

4. Discussion

As we all know, China ranks among the top in the global burden of tuberculosis.[1] Lymph node tuberculosis, as the second most common tuberculosis after pulmonary tuberculosis,[22] still has a rising incidence rate in China. At present, surgical resection of the lesion has been the gold standard for diagnosing lymph node lesions,[4] however, this standard itself has great limitations for diagnosis. Therefore, in our study, we recruited 101 patients with suspected cervical lymph node tuberculosis. Lesion tissues were obtained by ultrasound-guided puncture biopsy for traditional pathogen detection and high-throughput sequencing. We attempted to elucidate the diagnostic capabilities of clinical metagenomic next-generation sequencing and metagenomic third-generation sequencing in cervical lymph node tuberculosis.

We systematically evaluated the performance of culture, X-pert, metagenomic next-generation sequencing and metagenomic third-generation sequencing in the diagnosis of *M. tuberculosis*. Similar to many studies, culture showed low sensitivity (43.28 %).[23,24] X-pert, as one of the mainstream diagnostic methods currently, has higher

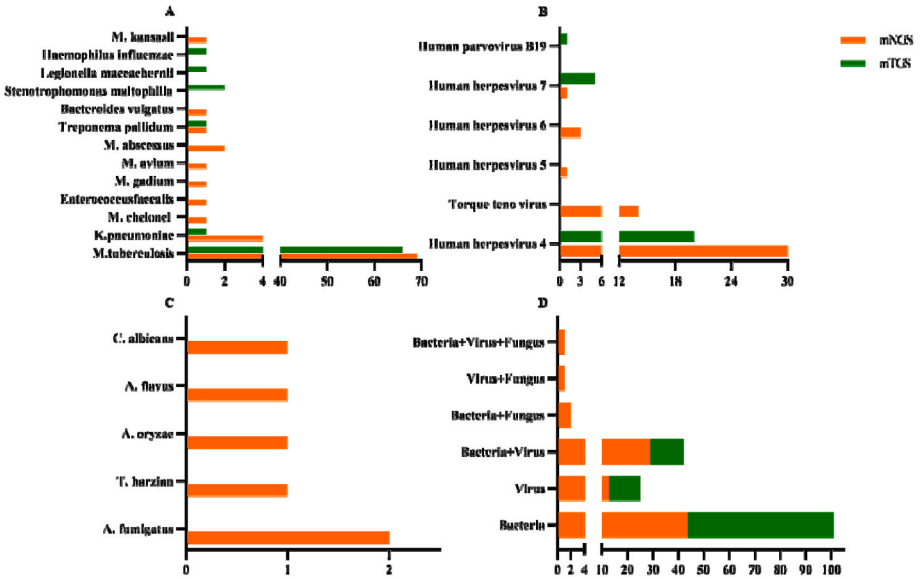

Fig. 4. Overview of pathogenic detection by high-throughput sequencing. (A) Distribution of bacteria; (B) Distribution of virus; (C) Distribution of fungus; (D) Distribution of infection type.

Table 4
The impact of anti-tuberculosis drugs on the results of the detection and the number of high-throughput sequencing reads.

	Group	Culture	X-pert	mNGS	mTGS
Positive rate/% (n/N)	Anti-tuberculosis therapy	6.67 % (1/15)	80.00 % (12/15)	86.67 % (13/15)	80.00 % (12/15)
	No anti-tuberculosis therapy	40.28 % (29/72)	48.61 % (35/72)	68.06 % (49/72)	65.28 % (47/72)
Reads/median (IQR)	Anti-tuberculosis therapy	/	/	71 (9–5811.5)	131 (23.25–2083.25)
	No anti-tuberculosis therapy	/	/	105 (17–666.5)	51 (16–641)

sensitivity (68.42 %). Because *M. tuberculosis* is an intracellular bacterium, its detection threshold is very low, even if only one read maps to this species.[9] In addition, lymph nodes are different from organs such as the lungs and are not exposed to the external environment, so it is naturally free from the colonization of microorganisms. In this case, as a non-biased detection method, mNGS and mTGS, not surprisingly, showed more excellent diagnostic performance (89.74 % and 82.89 %, Separately), both are significantly higher than X-pert and culture, which is consistent with previous reports.[12,25,26] Compare to mTGS, mNGS showed better sensitivity and specificity (100 % vs 95 %), but it was not statistically significant. mNGS and mTGS showed comparable ability in the diagnosis of cervical lymph node tuberculosis.

Although, in this study, combined with high-throughput sequencing and X-pert, we further improved the identification ability of tuberculosis, there are inevitably cases of false positives and false negatives. How to accurately identify active tissue and necrotized areas under ultrasound guidance may be the most influential factor in cervical lymph node tuberculosis. Besides, sample preprocessing, host removal methods, and library construction capabilities are all influencing factors.[27] By increasing the sequencing depth, it undoubtedly improves the quality of sequencing, but it will inevitably further increase the economic burden.[28]

Coinfections of *M. tuberculosis* and fungi often increase the difficulty of diagnosis and treatment in tuberculosis.[29,30] In this study, all 5 fungi were detected alone by mNGS. And *M. tuberculosis* and viral coinfection is the most common coinfection, which is similar to previous studies.[31,32] Most bacterial-viral coinfections are defined by mNGS (29/90). The accuracy of the Illumina platform may be higher than that of the Nanopore platform.[33] Third-generation sequencing, due to its longer read length, inevitably brings a higher probability of misreading, which may require deeper sequencing depth or more careful library preparation to compensate for it.[34]

Because Hangzhou Red Cross Hospital is a tuberculosis specialist hospital, more patients are referred to us. In addition, the treatment cycle of tuberculosis is relatively long. Therefore, many tuberculosis patients have used or are taking anti-tuberculosis drugs when they visit the hospital. Due to the fact that culture can only identify viable bacteria, the use of anti-tuberculosis drugs significantly reduces the positive rate of culture. However, it has no significant effect on the positive rate of X-pert and high-throughput sequencing, as found in previous studies.[25,35] For these patients, high-throughput sequencing avoids delaying treatment due to misdiagnosis.

In our study, we identified several cases of drug-resistant *M. tuberculosis* through culture-antimicrobial susceptibility test and X-pert, however, high-throughput sequencing did not report the drug resistance. To achieve this goal, it is necessary to increase sequencing depth,[36] improve the analysis process,[37] and increase the database related to drug resistance (The mechanism of tuberculosis resistance is different from that of the vast majority of pathogens).[33] Through technological optimization, developing targeted high-throughput sequencing may be the most cost-effective measure.

Although we have demonstrated the excellent diagnostic performance of metagenomic sequencing in cervical lymph node tuberculosis, this study still has some limitations. Due to the nature of Hangzhou Red Cross Hospital, our patients are mainly tuberculosis patients, and the range of pathogens is relatively small, so it is impossible to verify the diagnostic performance of metagenomic sequencing in a more complex pathogenic background. The sampling method of ultrasound-guided puncture biopsy greatly challenge the experience and skill of the operator and becomes the key factor directly affecting the final sequencing result. The sample type is relatively simple, and more sample types should be evaluated.

In conclusion, compared to traditional detection methods, mNGS and mTGS have better detection performance in cervical lymph node tuberculosis and show considerable diagnostic value, as well as, are less affected by previous drug use. mNGS identifies more potential pathogenic microorganisms than mTGS. All of these contribute to patients' access to rapid diagnosis and accurate treatment.

CRediT authorship contribution statement

Ting Lin: Conceptualization, Writing – original draft. **Yuehui Yu:** Conceptualization, Writing – original draft. **Jialei Luo:** Data curation. **Xinyi Yan:** Investigation, Data curation. **Yuxuan Qiu:** Formal analysis, Data curation. **Jiahui Tong:** Methodology. **Ying Wang:** Methodology, Conceptualization. **Xiangyun Huang:** Supervision, Methodology. **Dan Li:** Validation, Methodology. **Ying Zhang:** Writing – review & editing. **Gaoyi Yang:** Funding acquisition, Conceptualization.

References

1. Rodriguez-Takeuchi SY, Renjifo ME, Medina FJ. Extrapulmonary tuberculosis: Pathophysiology and imaging findings. *RadioGraphics*. 2019;39(7):2023–2037. https://doi.org/10.1148/rg.2019190109.
2. Park KH, Cho OH, Chong YP, et al. Post-therapy paradoxical response in immunocompetent patients with lymph node tuberculosis. *J Infect*. 2010;61(5):430–434. https://doi.org/10.1016/j.jinf.2010.08.011.
3. Cataño JC, Robledo J. Tuberculous lymphadenitis and parotitis. *Microbiol Spectr*. 2016;4(6). ARTN TNMI7-0008-201610.1128/microbiolspec.TNMI7-0008-2016.
4. Meng XY, Fu HX, Jia WA, Wang Y, Yang GY. A comparative study of ultrasound-guided puncture biopsy combined with histopathology and Xpert MTB/RIF in the diagnosis of lymph node tuberculosis. *Front Public Health*. 2023;10. ARTN 102247010.3389/fpubh.2022.1022470.
5. Li N, Cai QQ, Miao Q, Song ZS, Fang Y, Hu BJ. High-throughput metagenomics for identification of pathogens in the clinical settings. *Small Methods*. 2021;5(1). ARTN 200079210.1002/smtd.202000792.
6. Kisand V, Valente A, Lahm A, Tanet G, Lettieri T. Phylogenetic and functional metagenomic profiling for assessing microbial biodiversity in environmental monitoring. *PLoS One*. 2012;7(8). ARTN e4363010.1371/journal.pone.0043630.
7. Li SY, Tong J, Liu Y, Shen W, Hu P. Targeted next generation sequencing is comparable with metagenomic next generation sequencing in adults with pneumonia for pathogenic microorganism detection. *J Infect*. 2022;85(5):E127–E129. https://doi.org/10.1016/j.jinf.2022.08.022.
8. Zheng Y, Qiu XJ, Wang T, Zhang J. The diagnostic value of metagenomic next-generation sequencing in lower respiratory tract infection. *Front Cell Infect Microbiol*. 2021;11. ARTN 69475610.3389/fcimb.2021.694756.
9. Zhao N, Cao JB, Xu JY, et al. Targeting RNA with next- and third-generation sequencing improves pathogen identification in clinical samples. *Adv Sci*. 2021;8(23). ARTN 210259310.1002/advs.202102593.
10. Xiao GH, Cai Z, Guo QL, et al. Insights into the unique lung microbiota profile of pulmonary tuberculosis patients using metagenomic next-generation sequencing. *Microbiol Spectr*. 2022;10(1).
11. Gould CV, Free RJ, Bhatnagar J, et al. Transmission of yellow fever vaccine virus through blood transfusion and organ transplantation in the USA in 2021. *Lancet Microbe*. 2023;4(9):711–721. https://doi.org/10.1016/S2666-5247(23)00170-2.
12. Li YG, Jiao MF, Liu Y, Ren ZG, Li A. Application of metagenomic next-generation sequencing in infection. *Front Med*. 2022;9. ARTN 80271910.3389/fmed.2022.802719.
13. Xiang ZB, Leng EL, Cao WF, et al. A systematic review and meta-analysis of the diagnostic accuracy of metagenomic next-generation sequencing for diagnosing tuberculous meningitis. *Front Immunol*. 2023;14. ARTN 122367510.3389/fimmu.2023.1223675.
14. Eckert SE, Chan JZM, Houniet D, Breuer J, Speight G, Consortium P. Enrichment by hybridisation of long DNA fragments for Nanopore sequencing. *Microb Genom*. 2016;2(9). ARTN 00008710.1099/mgen.0.000087.

15. Smith C, Halse TA, Shea J, et al. Assessing Nanopore sequencing for clinical diagnostics: a comparison of Next-Generation Sequencing (NGS) methods for. *J Clin Microbiol*. 2021;59(1). ARTN e00583-2010.1128/JCM.00583-20.
16. Li Y, Li GY, Han DX, et al. Study on the clinical indications for plasma as an alternative to the bronchoalveolar lavage fluid metagenomic next-generation sequencing test to detect consistent pathogens for septic patients in intensive care units. *J Infect*. 2022;85(4):442–445. https://doi.org/10.1016/j.jinf.2022.07.016.
17. Miller S, Naccache SN, Samayoa E, et al. Laboratory validation of a clinical metagenomic sequencing assay for pathogen detection in cerebrospinal fluid. *Genome Res*. 2019;29(5):831–842. https://doi.org/10.1101/gr.238170.118.
18. Muni S, Rajpal K, Kumar R, et al. Identification of fungal isolates in patients with pulmonary tuberculosis treated at a tertiary care hospital. *Cureus*. 2023. https://doi.org/10.7759/cureus.37664.
19. Luo W, He YB, Xu JH, et al. Comparison of third-generation sequencing technology and traditional microbiological detection in pathogen diagnosis of lower respiratory tract infection. *Discov Med*. 2023;35(176):332–342. https://doi.org/10.24976/Discov.Med.202335176.34.
20. Zhao MN, Zhang YY, Chen L, et al. Nanopore sequencing of infectious fluid is a promising supplement for gold-standard culture in real-world clinical scenario. *Front Cell Infect Microbiol*. 2024;14. ARTN 133078810.3389/fcimb.2024.1330788.
21. Chinese expert consensus on metagenomics next-generation sequencing application on pathogen detection of infectious diseases. *Chin J Lab Med*. 2021;44. https://doi.org/10.3760/cma.j.cn114452-20201026-00794.
22. Handa U, Mundi I, Mohan S. Nodal tuberculosis revisited: a review. *J Infect in Dev Ctries*. 2012;6(1):6–12. https://doi.org/10.3855/jidc.2090.
23. Liu Y, Wang HF, Li YG, Yu ZJ. Clinical application of metagenomic next-generation sequencing in tuberculosis diagnosis. *Front Cell Infect Microbiol*. 2023;12. ARTN 98475310.3389/fcimb.2022.984753.
24. Li YL, Bian WT, Wu SP, Zhang J, Li D. Metagenomic next-generation sequencing for complex detection: a meta-analysis. *Front Public Health*. 2023;11. ARTN 122499310.3389/fpubh.2023.1224993.
25. Liu X, Chen YL, Ouyang H, et al. Tuberculosis diagnosis by metagenomic next-generation sequencing on bronchoalveolar lavage fluid: a cross-sectional analysis. *Int J Infect Dis*. 2021;104:50–57. https://doi.org/10.1016/j.ijid.2020.12.063.
26. Jin X, Li J, Shao MY, et al. Improving suspected pulmonary infection diagnosis by bronchoalveolar lavage fluid metagenomic next-generation sequencing: a multicenter retrospective study. *Microbiol Spectr*. 2022;10(4). https://doi.org/10.1128/spectrum.02473-21.
27. Gu W, Miller S, Chiu CY. Clinical metagenomic next-generation sequencing for pathogen detection. *Annu Rev Pathol*. 2019;14(14):319–338. https://doi.org/10.1146/annurev-pathmechdis-012418-012751.
28. Simner PJ, Miller S, Carroll KC. Understanding the promises and hurdles of metagenomic next-generation sequencing as a diagnostic tool for infectious diseases. *Clin Infect Dis*. 2018;66(5):778–788. https://doi.org/10.1093/cid/cix881.
29. Fang WJ, Zhang L, Liu J, et al. Tuberculosis/cryptococcosis co-infection in China between 1965 and 2016. *Emerg Microb Infect*. 2017;6. ARTN e7310.1038/emi.2017.61.
30. Willcocks S, Wren BW. Shared characteristics between and fungi contribute to virulence. *Future Microbiol*. 2014;9(5):657–668. https://doi.org/10.2217/fmb.14.29.
31. Liang MY, Fan YM, Zhang DM, et al. Metagenomic next-generation sequencing for accurate diagnosis and management of lower respiratory tract infections. *Int J Infect Dis*. 2022;122:921–929. https://doi.org/10.1016/j.ijid.2022.07.060.
32. Peng JM, Du B, Qin HY, Wang Q, Shi Y. Metagenomic next-generation sequencing for the diagnosis of suspected pneumonia in immunocompromised patients. *J Infect*. 2021;82(4):22–27. https://doi.org/10.1016/j.jinf.2021.01.029.
33. Hall MB, Rabodoarivelo MS, Koch A. Evaluation of Nanopore sequencing for Mycobacterium tuberculosis drug susceptibility testing and outbreak investigation: a genomic analysis (vol 4, pg 84, 2022). *Lancet Microbe*. 2023;4(2):E72–E. https://doi.org/10.1016/S2666-5247(22)00381-0.
34. Meslier V, Quinquis B, Da Silva K, et al. Benchmarking second and third-generation sequencing platforms for microbial metagenomics. *Sci Data*. 2022;9(1). ARTN 69410.1038/s41597-022-01762-z.
35. Huang ZD, Zhang ZJ, Yang B, et al. Pathogenic detection by metagenomic next-generation sequencing in osteoarticular infections. *Front Cell Infect Microbiol*. 2020;10. ARTN 47110.3389/fcimb.2020.00471.
36. Cabibbe AM, Spitaleri A, Battaglia S, et al. Application of targeted next-generation sequencing assay on a portable sequencing platform for culture-free detection of drug-resistant tuberculosis from clinical samples. *J Clin Microbiol*. 2020;58(10). ARTN e00632-2010.1128/JCM.00632-20.
37. Grobbel HP, Merker M, Köhler N, et al. Design of multidrug-resistant tuberculosis treatment regimens based on DNA sequencing. *Clin Infect Dis*. 2021;73(7):1194–1202. https://doi.org/10.1093/cid/ciab359.