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# Evaluation of targeted sequencing for pathogen identification in bone and joint infections: a cohort study from China

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## Abstract

**Purpose** Bone and joint tuberculosis (BJTB) is a distinct variant of tuberculosis in which clinical diagnosis often leads to relative misdiagnosis and missed diagnoses. This study aimed to evaluate the diagnostic accuracy of the targeted nanopore sequencing (TNPseq) assay for BJTB patients in China.

**Method** The study enrolled a cohort of 163 patients with suspected BJTB. Diagnostic testing was performed using the TNPseq assay on samples including punctured tissue, pus, and blood. The diagnostic accuracy of the TNPseq assay was then compared with that of the T-SPOT and Xpert MTB/RIF assays.

**Result** TNPseq exhibited superior performance in terms of accuracy, demonstrating a sensitivity of 76.3% (95% CI: 71.0–81.6%) and a specificity of 98.8% (95% CI: 93.5–100%) in clinical diagnosis. When evaluated against a composite reference standard, TNPseq demonstrated a sensitivity of 74.4% (95% CI: 69.3–79.5%) and a specificity of 98.8% (95% CI: 93.7–100%). These results exceed the performance of both the T-SPOT and Xpert MTB/RIF tests. Notably, TNPseq demonstrated high specificity and accuracy in puncture specimens, with a sensitivity of 75.0% (95% CI: 70.2–79.8%) and a specificity of 98.3% (95% CI: 92.7–100%), as well as in pus samples, with a sensitivity of 83.3% (95% CI: 78.6–88.1%) and a specificity of 100% (95% CI: 100–100%). Additionally, TNPseq facilitated the detection of mixed infection scenarios, identifying 20 cases of bacterial-fungal co-infection, 17 cases of bacterial-viral co-infection, and two cases of simultaneous bacterial-fungal-viral co-infection.

**Conclusion** TNPseq demonstrated great potential in the diagnosis of BJTB due to its high sensitivity and specificity. The ability of TNPseq to diagnose pathogens and detect drug resistance genes can also guide subsequent treatment. Expanding the application scenarios and scope of TNPseq will enable it to benefit more clinical treatments.

**Keywords** Diagnosis, Infection, Targeted nanopore sequencing, Bone and joint, Tuberculosis, Xpert MTB/RIF

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## Background

The global adult mortality rate for tuberculosis (TB) remains alarmingly high, making it the second most deadly disease in the world [1]. Bone and joint tuberculosis (BJTB) is a unique manifestation of TB characterized by aggressive invasion of *Mycobacterium tuberculosis* (M.tb) into the skeletal system, joints, and surrounding tissues. This can lead to the formation of abscesses and ulcerations at the affected sites [2]. Clinical identification of BJTB is challenging due to its insidious onset, non-specific early symptoms, and equivocal radiologic findings, often leading to misdiagnosis and underreporting of cases [3]. This underscores the critical need for improved diagnostic strategies to accurately identify BJTB.

To address this diagnostic challenge, researchers have sought to increase the detection rate of bone and joint tuberculosis (BJTB). While the traditional method of culturing M.tb has been considered the benchmark, it is hampered by its lengthy process and relatively low rate of successful detection [4, 5]. In 1989, Hance introduced polymerase chain reaction (PCR) technology as a means of detecting tuberculosis, significantly streamlining the diagnostic process [6]. While PCR technology offers significant advantages, it also has its drawbacks. Problems such as false negatives and other errors can occur due to factors such as primer selection, probe design, and overall assay configuration [7]. The  $\gamma$ -interferon release assay, including the T-lymphocyte spot (T-SPOT) assay, which is based on cellular immunity, has also been developed [8]. However, the T-SPOT assay is susceptible to interference from cross-reactive antigens and immune complexes, as well as the influence of patients' prior injections of purified protein derivatives [4, 8]. These issues remain unresolved in the clinical setting. Therefore, the development of a rapid diagnostic method that reduces the risk of misdiagnosis and missed diagnoses would be of significant benefit to patients.

Next-generation sequencing (NGS) technology has been adopted by clinical laboratories worldwide in recent years [9]. Hall has validated the use of nanopore technology for genotyping M.tb, drug susceptibility testing, and outbreak investigations [10]. However, the practical application of whole-genome sequencing in clinical settings is currently constrained by the need for an initial TB culture to obtain a sufficient amount of bacteria for sequencing [11]. In contrast, targeted nanopore sequencing (TNPseq) allows direct analysis of clinical samples without the need for culture [12]. By taking advantage of the exceptionally long read lengths of TNPseq, this method offers the potential for rapid and early diagnosis of TB, making it a viable solution for large-scale, rapid, and clinically applicable sequencing [13]. Sun et al. evaluated the efficacy of TNPseq technology for the detection of M.tb in bronchoalveolar lavage specimens. In

a cohort of 58 patients with suspected pulmonary TB, using clinical composite diagnosis as the reference standard, TNPseq demonstrated a sensitivity of 89.6% (95% CI: 77.3–96.5) [14]. While TNPseq has been widely used to diagnose pulmonary TB, its utility in samples with low bacterial loads remains less understood in terms of diagnostic accuracy and appropriateness. Chan and colleagues evaluated a nanopore sequencing approach for the direct identification of TB and analysis of drug resistance in 23 clinical samples with low bacterial loads. Although their diagnostic results were less favorable than those reported by Sun et al., their research laid the groundwork for the use of TNPseq in low M.tb specimens [15]. Challenges in BJTB sample collection and limited experimental data have hindered the application of TNPseq, highlighting the need for further research into its use in BJTB patients.

In this study, we conducted TNP sequencing on 163 patients and compared the results of TNPseq with the GeneXpert MTB/RIF assay and T-SPOT. We evaluated the potential of TNPseq in pus and puncture specimens for pathogen identification and drug resistance insights to improve BJTB diagnosis, reduce misdiagnosis, and enable timely, targeted patient care.

## Methods and materials

### Study design and participant enrollment

This study was conducted from July 2022 to June 2023 at the Orthopedic Hospital, Public Health Clinical Center, Shandong Province, China, with 234 clinical specimens suspected of BJTB. Participants presented with BJTB-related signs and symptoms, and surgical specimens were collected. The protocol (No. GWLCZXEC-AF-03-1-1) was ethically approved, and all patients gave informed consent.

The study inclusion criteria were as follows: (1) patients suspected of having BJTB on admission to hospital, with typical clinical features of TB and characteristic radiological findings on x-ray, computed tomography (CT) and magnetic resonance imaging (MRI); (2) patients between 15 and 85 years of age; (3) patients from whom at least one sample of infected site pus and one sample of blood were obtained within 1 h before surgery; (4) patients without tissue samples had to have at least one sample of infected site tissue; (5) a minimum of 150 patients were to be included.

Study exclusion criteria were as follows: (1) individuals who had received anti-TB treatment in the previous 2 months; (2) individuals with incomplete case information; (3) individuals with incomplete clinical laboratory test results; (4) individuals with combined infections of other organs or systems; (5) individuals who did not provide informed consent.

### Sample collection

**Pus specimens:** Specimens were obtained intraoperatively to collect pus from the joint space within the patient's lesion, necessitated by the presence of an abscess. **Punctured tissue:** When pus was not easily obtained from patients, the puncture technique was used to obtain samples from the necrotic bone or granuloma surrounding the lesion site. **Peripheral blood:** Approximately 10 mL of peripheral venous blood was collected from each patient under strict aseptic conditions. The collected pus samples were gently vortexed and then aseptically divided into two undiluted aliquots. Similarly, the punctured tissue was divided equally into two unbiased segments. One segment was sent to the microbiology laboratory of the Orthopedic Hospital of the Shandong Provincial Public Health Clinical Center for bacterial culture analysis. The second segment was immediately sent to Hangzhou ShengTing Biotechnology Co. Ltd. in Hangzhou, China for TNPseq processing using cold chain logistics. Both laboratories followed the "National Clinical Testing Practice for Pathogen Detection in Clinical Microbiology Laboratory" protocol for standard analytical procedures.

### Clinical microbiology test

#### Culture

Two milliliters (2 mL) of samples were first digested and decontaminated with N-acetyl-L-cysteine/NaOH as described in previous research [5]. The precipitate was concentrated by centrifugation and the supernatant was discarded. Phosphate buffered saline (pH 6.8) was added to the precipitate to a final volume of 2.5 mL. Then 0.5 mL of this mixture was added to a 7 mL MGIT tube. The 7H9 Middlebrook broth (BD-Difco, USA) in the MGIT tube contained nutrients necessary for the growth of *M.tb*, while the BBL MGIT PANTA antibiotic mixture (BD-Difco, USA) provided an environment conducive to the selective growth of *M.tb* while inhibiting the growth of other bacteria. Tubes were then incubated in the MGIT 960 TB System (BD-Difco, USA) for automated reporting.

#### T-SPOT assay

Following the protocol of Patel et al. [8], 2–3 mL of peripheral blood was collected. The reagent provided in the T-SPOT kit (Oxford Immunotec Ltd., UK) was added and the mixture was incubated at 37 °C for 20 min. The supernatant was carefully removed with a special pipette and transferred to antigen-coated discs. After the incubation period, the antigen plate was removed from the incubator and placed in a specialized plate reader. The output of the plate reader was used to determine the presence and extent of *M.tb*-specific T-cell responses.

### Molecular detection test

#### Sample preparation

**Pus specimens:** 1 mL of the sample was added to a tube containing 1 mL of phosphate buffered saline (PBS) and mixed thoroughly. **Punctured tissue:** Tissue block was cut into small pieces and added to 2 mL of PBS. The tissue was then ground to a homogeneous suspension using a tissue grinder. **Peripheral blood:** Blood was first centrifuged, then mononuclear cells were isolated. 2 mL of the top layer of plasma was transferred to a new pretreated tube and the sample was maintained at 30 °C.

#### Xpert MTB/RIF assay

Following the methodology described in previous research [3], 1 mL of sample suspension was transferred to a separate tube to which 2 mL of Xpert Sample Reagent was added. The contents were vortexed for at least 10 s and incubated at 20 °C for 15 min. After incubation, 2 mL of the mixture was transferred to an Xpert Reaction Cartridge, which was then inserted into the Xpert instrument. Assay results were automatically generated within 2 h.

#### TNPseq assay

**Sample processing** The samples were centrifuged and then digested with proteinase K and lysozyme. They were then thoroughly ground with zirconia beads for 1 min to break down the cell walls. After grinding, lysis was achieved by adding a lysozyme solution, and the resulting lysate was used for nucleic acid extraction using the QIAamp DNA Microbiome Kit (QIAGEN, Canada). A negative control consisting of blank elution buffer was included for the extracted nucleic acids. The concentration of the extracted DNA was measured using the Qubit dsDNA Quantification Assay Kit (ThermoFisher, USA). For detection, the bacterial 16 S rRNA gene was targeted with universal primers 27 F/1492R, while the fungal ITS1/2 gene was amplified by PCR using ITS1/4 primers. PCR was performed on an ABI 2720 thermocycler under the following conditions: initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 62 °C for 60 s, and 72 °C for 60 s, with a final extension at 72 °C for 3 min. PCR products were then purified and quantified using the Invitrogen Qubit 4 Fluorometer in preparation for library construction and subsequent TNPseq sequencing.

**Library preparation** Nanopore Barcode PCR was performed on the above PCR products according to the PCR Barcode Expansion Pack 1–96 (EXP-PBC096) to generate the sequencing libraries. After initial start-up of the sequencing chip, approximately 100 ng of the library pools were loaded into the nanopore flow cell for sequencing on the GridION platform. MinKNOW version 2.0 software

was used to facilitate data output for base calling. Barcode demultiplexing was then performed using Porechop.

**Identification of pathogen and drug resistance genes** The EPI2ME platform was used through the 16 S workflow. Raw data was converted into Fastq file using Guppy base calling software. Reads less than 200 bp or greater than 2000 bp with a quality score of  $Q < 9$  were filtered out, and the remaining high quality reads were aligned to all targets and potential etiologies using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). Pathogens were categorized at the species level based on coverage and identity. In general, the top 10 microorganisms sorted by aligned reads with a relative abundance score greater than 0.5% were classified as pathogens and further evaluated. *M.tb* is considered positive if at least one sequence is mapped to a species or genus. All these steps in the TNPseq assay were performed according to our previous report [16]. The TNPseq assay allows simultaneous detection of multiple pathogens and drug resistance. It accurately identifies a wide range of important pathogens, such as *M.tb* and non-tuberculous mycobacteria, and detects 21 drug resistance genes, including *rpoB* for rifampicin and *katG* for isoniazid. Table S5 shows the range of resistance genes that can be identified by TNPseq. For more details on this procedure, see the study by Liu et al. [17].

#### Serum immunological test

Following the protocol established by Patel et al. [8], patient blood samples were collected into EDTA anticoagulant tubes. After centrifugation at 1000 g for 10 min, 25  $\mu$ L of plasma was aliquoted into assay tubes. An equal volume of buffer, as required by the flow cytometer, was added along with microbeads conjugated to antibodies against IL-6, IFN- $\gamma$ , IL-10, and other markers, as well as the appropriate detection antibodies. Streptavidin-phycoerythrin (SA-PE) conjugated to allophycocyanin (APC) was then added and the mixture was analyzed using a Navios flow cytometer (Beckman, USA). Upon completion of the flow cytometric analysis, the calibration standards and sample data were imported into the LEGENDplex 8.0 software for analysis, which provided the concentrations of the respective cytokines for each sample.

#### Patient categories

Clinical diagnostic criteria included a comprehensive evaluation that included a detailed medical history, serologic testing, pathologic examination of tissue samples showing tuberculous changes (e.g., chronic granulomatous inflammation with caseous necrosis), and imaging (-ray, CT, or MRI) showing characteristic features of BJTB.

According to the reference with some modifications [3], the composite reference standard (CRS) used in this study consisted of clinical diagnosis and culture results. Patients were classified into three categories based on the CRS criterion: Confirmed BJTB: Patients who were clinically diagnosed with BJTB and had a positive culture result; Probable BJTB: Patients with a positive result in either clinical diagnosis or pathogen culture, while the other was negative; Non-BJTB: Patients with negative clinical diagnosis and pathogen culture results. Clinical diagnosis and CRS were used as reference criteria to evaluate the diagnostic value of TNPseq test for BJTB.

#### Statistical analysis

Clinical and experimental data were initially recorded in an Excel spreadsheet and analyzed for the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of TNPseq, Xpert MTB/RIF, T-SPOT, and culture using SPSS 24.0. Descriptive statistics included mean, standard deviation (SD), median, and interquartile range (IQR). Comparisons were performed with the  $\chi^2$  test, and a p-value less than 0.05 was deemed statistically significant. Data visualization was performed using Origin 2022.

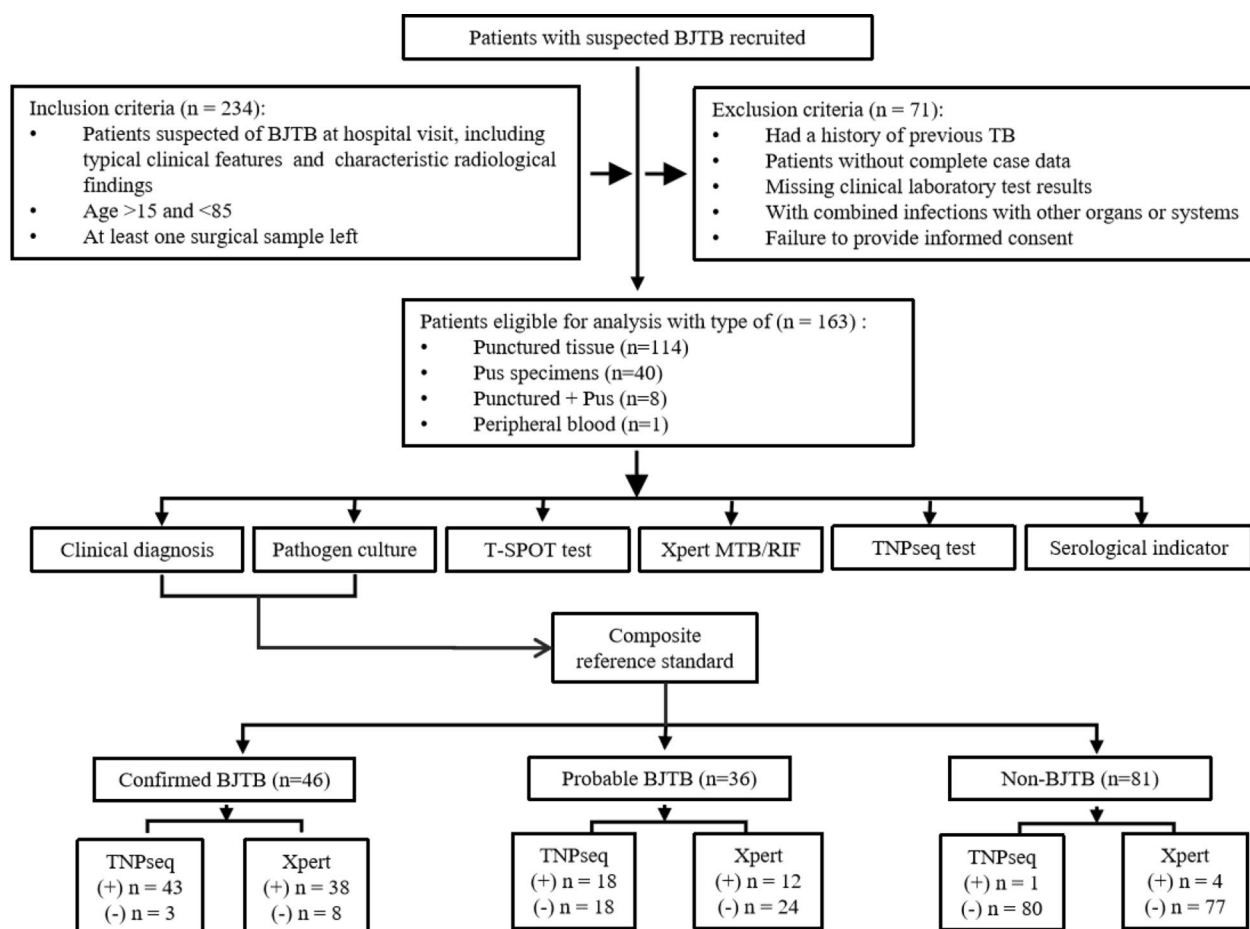
## Results

#### Patient characteristics

After applying the exclusion criteria, 71 patients were excluded, leaving 163 patients for analysis (Fig. 1). We collected puncture tissue samples from 114 patients, pus samples from 40 patients, mixed samples of puncture tissue and pus from 8 patients, and a single peripheral blood sample from 1 patient. Of these clinical specimens, 102 (62.5%) were from male patients and 61 (37.4%) were from female patients. The mean age for males was  $52.9 \pm 16.5$  years and for females  $60.1 \pm 14.4$  years. The demographic and clinical characteristics of the study population are shown in Table 1, and the diagnoses and specific sources of lesions are shown in Table S1.

According to the clinical diagnosis, 80 patients (49.1%) were identified with BJTB, whereas 83 patients (50.9%) were determined not to have the disease. Of the 80 patients clinically diagnosed with BJTB, 38 (47.5%) had positive culture results, 50 (62.5%) had positive Xpert MTB/RIF test results, 58 (72.5%) had positive T-SPOT test results, and 61 (76.25%) had positive TNPseq results.

Using CRS as the reference standard, 81 patients (49.7%) were classified as not having BJTB, 46 patients (28.2%) were confirmed to have BJTB, and 36 patients (22.1%) were classified as likely to have BJTB.



**Fig. 1** Flowchart of the study population BJTB: Bone and joint tuberculosis; TB: tuberculosis; TNPseq: Targeted nanopore sequencing; (+) n: Positive cases number; (-) n: Negative cases number

### Efficacy of TNPseq assay under different reference standards

Using clinical diagnosis as the reference standard, TNPseq yielded a sensitivity of 76.3% (95% CI: 71.0–81.6%), a specificity of 98.8% (95% CI: 93.5–100%), a positive predictive value (PPV) of 98.4% (95% CI: 93.1–100%), a negative predictive value (NPV) of 81.2% (95% CI: 75.9–86.5%), a positive likelihood ratio (PLR) of 63.6 (95% CI: 58.3–68.9), a negative likelihood ratio (NLR) of 0.239 (95% CI: 0.215–0.263), and an area under the curve (AUC) of 0.875 (95% CI: 0.816–0.934). These results are detailed in Table 2 and illustrated in Fig. 2. When employing CRS as the standard, TNPseq performance metrics included sensitivity of 74.4% (95% CI: 69.3–79.5%), specificity of 98.8% (95% CI: 93.7–100%), PPV of 98.4% (95% CI: 93.3–100%), NPV of 79.2% (95% CI: 74.1–84.3%), PLR of 62.0 (95% CI: 56.9–67.1), NLR of 0.2259 (95% CI: 0.235–0.283), and AUC of 0.866 (95% CI: 0.805–0.926). These results were not significantly different from those obtained using clinical diagnosis. The sensitivity and specificity of Xpert were 62.5% (95% CI: 57.2–67.8%) and

95.2% (95% CI: 89.9–100%), respectively. T-SPOT had a sensitivity of 72.5% (95% CI: 67.2–77.8%) and a specificity of 81.9% (95% CI: 76.6–87.2%). Compared to the other two methods, TNPseq consistently showed superior performance in both sensitivity and specificity.

### Correlation between serology and TNPseq results

Using the CRS criteria, serum samples from 46 patients with confirmed BJTB and 81 patients without BJTB were collected for analysis. The results showed significantly elevated serum levels of IL-6, IFN- $\gamma$ , and IL-10 in the confirmed BJTB patients compared with the non-BJTB cases, as shown in Fig. S3 and Table S3. However, case 56 had unique characteristics. Despite being negative for comprehensive diagnosis and negative for all other diagnostic tests, TNPseq yielded a positive result. In addition, the serum levels of IL-6, IFN- $\gamma$ , and IL-10 in this patient were significantly different from those of non-BJTB patients.

**Table 1** Characteristics of study participants stratified by hospital

Characteristics	Value
Median age (interquartile range, IQR)	59 (47, 68)
Gender (n, %)	
Men	102 (62.5%)
Women	61 (37.4%)
BMI (interquartile range, IQR)	23.36 (21, 26.3)
Underweight (BMI < 18.5)	5
Combined diabetes	
Yes	11
No	152
Co-HIV infection	
Yes	1
No	162
Cardinal symptom	
Pain	134
Fever	12
Swell	6
Night sweat	2
Other	9
History of previous tuberculosis	
Yes	0
No	163
Sample type	
Puncture tissue	114
Pus specimens	40
Peripheral blood	1
Puncture tissue + Pus specimens	8

**Efficacy of TNPseq assay for different sample types**

The efficacy of TNPseq for different sample types is compared in Table 3. Mixed specimens of pus and punctured tissue were obtained from a single patient during a single surgical procedure and underwent independent TNPseq analysis. The positive criterion for patients providing mixed infection specimens was defined as follows: If both samples showed positive bacterial presence, it was considered positive; otherwise, it was classified as negative. Using clinical diagnosis as the reference standard, TNPseq demonstrated a sensitivity of 75.0% (95% CI: 70.2–79.8%) and specificity of 98.3% (95% CI: 92.7–100%) in swab specimens. In pus specimens, TNPseq showed a sensitivity of 83.3% (95% CI: 78.6–88.1%) and a specificity of 100% (95% CI: 100–100%). Notably, the sensitivity of TNPseq for mixed samples was significantly reduced to 50% (95% CI: 45.2–54.8%), while the specificity remained high at 100% (95% CI: 100–100%). In addition, blood samples were obtained from a patient diagnosed as non-BJTB who also tested negative for TNPseq.

**Efficacy of TNPseq assay for detection of polymicrobial infections**

The predominant microbial species detected by the TNPseq assays are shown in Fig. S1. Of the 163 samples

cultured, 65 were confirmed to contain M.tb. In addition, fungi with thick cell walls were detected, predominantly *Candida albicans* (11 cases) and *Aspergillus* (4 cases, not shown), as well as low-biomass viruses such as human herpesvirus I (3 cases, not shown). The TNPseq method demonstrated the ability to specifically identify nontuberculous mycobacteria (NTM), including intracellular mycobacteria and pyogenic mycobacteria. In addition, as shown in Fig. S2, the TNPseq assay was able to identify a significant number of polymicrobial infections caused by multiple pathogens. Bacterial-fungal infections ( $n=20$ ) were the most common type of polymicrobial infection, followed by bacterial-viral infections ( $n=17$ ). Furthermore, there were six cases of simultaneous bacterial-fungal-viral polymicrobial infections.

**Efficacy of TNPseq assay in detecting drug resistance genes**

As detailed in Table S2, TNPseq was used to screen 163 BJTB patients for drug resistance genes. For  $\beta$ -lactam antibiotics, TNPseq identified a total of 9 resistance genes in different bacteria: *E. coli* (blaCTX-M and blaTEM), *K. pneumoniae* (blaSHV and blaNDM), *H. influenzae* (bla-TEM), and *A. baumannii* (blaSHV). For methicillin antibiotics, TNPseq detected 3 resistance genes in *S. aureus* (mecA) and *S. hominis subsp. hominis* (mecA). In addition, one patient was found to be resistant to rifampicin.

**Discussion**

BJTB is known for its insidious onset and suboptimal detection accuracy, posing a significant challenge to clinical diagnosis [1–4]. This study included 163 patients with suspected BJTB admitted between July 2022 and June 2023. Regardless of whether clinical diagnosis or CRS was used as the reference standard, the sensitivity and specificity of Xpert MTB/RIF and T-SPOT tests were lower compared to TNPseq, indicating that TNPseq provided the highest sensitivity and specificity among the three methods [18]. These findings are consistent with the study by Tafess et al., which also highlighted the superior performance of TNPseq in tuberculosis detection [19]. Nanopore sequencing, which relies on longer sequencing reads, offers more accurate pathogen detection compared to non-sequencing based assays. Gu et al. used the GenoType MTBDRplus assay for rapid diagnosis of BJTB, but its sensitivity (72%, 36/50) was lower than that of TNPseq [1]. In addition, TNPseq is designed with targeted panels specific for clinically relevant pathogens, which further improves diagnostic accuracy. Therefore, we believe that TNPseq is a valuable tool for improving the efficiency of clinical diagnostics.

Serum factors in blood tests serve as key indicators of the host inflammatory response and are critical in the diagnosis of tuberculosis (TB) [3, 11]. Typically, upon TB infection, the inflammatory response is enhanced,

**Table 2** Diagnostic efficacy of the TNPseq assay

Items		Clinical diagnosis	CRS <sup>a</sup>
TNPseq	Sensitivity% (95% CI <sup>b</sup> )	76.3 (71.0–81.6)	74.4 (69.3–79.5)
	Specificity% (95% CI)	98.8 (93.5–100)	98.8 (93.7–100)
	PPV% (95% CI) <sup>c</sup>	98.4 (93.1–100)	98.4 (93.3–100)
	NPV% (95% CI) <sup>d</sup>	81.2 (75.9–86.5)	79.2 (74.1–84.3)
	PLR (95% CI) <sup>e</sup>	63.6 (58.3–68.9)	62.0 (56.9–67.1)
	NLR (95% CI) <sup>f</sup>	0.239 (0.215–0.263)	0.259 (0.235–0.283)
	AUC (95% CI) <sup>g</sup>	0.875 (0.816–0.934)	0.866 (0.805–0.926)
Xpert MTB/RIF	Sensitivity% (95% CI)	62.5 (57.2–67.8)	61.0 (55.9–66.1)
	Specificity% (95% CI)	95.2 (89.9–100)	95.1 (90.0–100)
	PPV% (95% CI)	92.6 (87.3–97.9)	92.6 (87.5–97.7)
	NPV% (95% CI)	72.5 (67.2–77.8)	70.6 (65.5–75.7)
	PLR (95% CI)	13.0 (12.4–13.7)	12.4 (11.8–13.1)
	NLR (95% CI)	0.394 (0.370–0.418)	0.410 (0.386–0.434)
	AUC (95% CI)	0.788 (0.716–0.860)	0.780 (0.706–0.854)
T-SPOT	Sensitivity% (95% CI)	72.5 (67.2–77.8)	70.7 (65.6–75.8)
	Specificity% (95% CI)	81.9 (76.6–87.2)	81.5 (76.4–86.6)
	PPV% (95% CI)	79.5 (74.2–84.8)	79.5 (74.4–84.6)
	NPV% (95% CI)	75.6 (70.3–80.9)	73.3 (68.2–78.4)
	PLR (95% CI)	4.01 (3.93–4.08)	3.82 (3.74–3.90)
	NLR (95% CI)	0.336 (0.312–0.359)	0.359 (0.336–0.384)
	AUC (95% CI)	0.772 (0.698–0.847)	0.761 (0.685–0.837)

a: Composite reference standard

b: Confidence interval

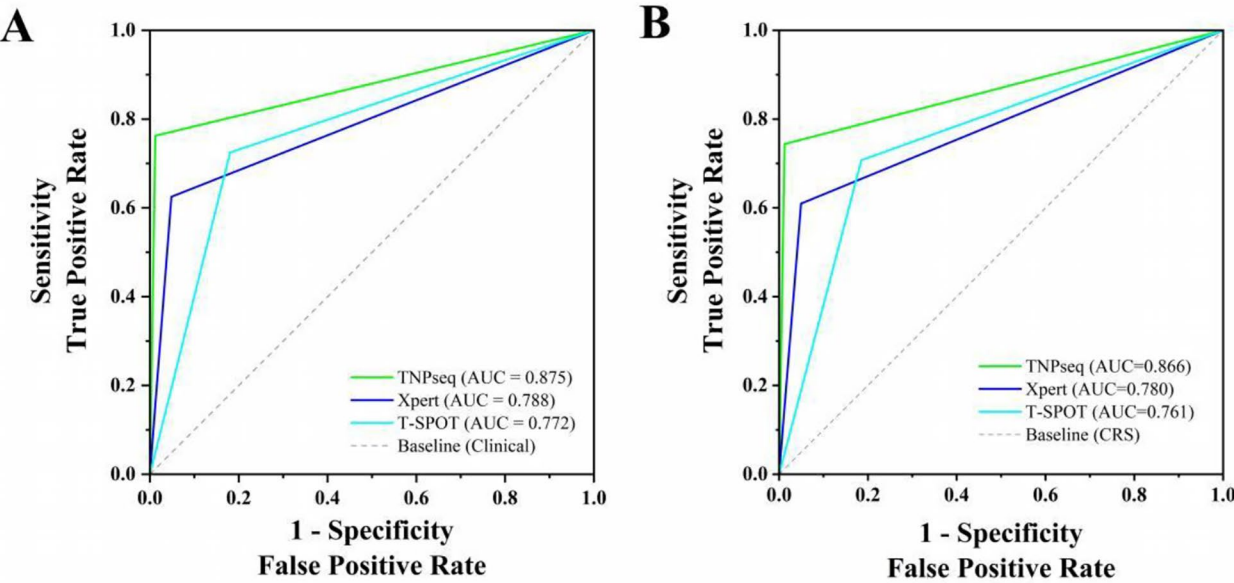
c: Positive predictive value

d: Negative predictive value

e: Positive likelihood ratio

f: Negative likelihood ratio

g: Area under curve



**Fig. 2** Receiver Operator Characteristic (ROC) curves of TNPseq assay compared with Xpert and T-SPOT **(A)** When clinical diagnosis was used as the reference standard, the Area Under Curve (AUC) of TNPseq was 0.875 (95% CI: 0.816–0.934); **(B)** When composite reference standard was used, the AUC of TNPseq was 0.866 (95% CI: 0.805–0.926) (Print in color)

**Table 3** Comparison of different sample types

Samples	Clinical diagnosis		CRS <sup>a</sup>	
	Sensitivity% (95% CI <sup>b</sup> )	Specificity% (95% CI)	Sensitivity% (95% CI)	Specificity% (95% CI)
Punctured tissue (n = 114)	75.0 (70.2–79.8)	98.3 (92.7–100)	72.2 (67.5–76.9)	98.3 (92.7–100)
Pus specimens (n = 40)	83.3 (78.6–88.1)	100 (100–100)	83.3 (78.6–88.1)	100 (100–100)
Punctured tissue + Pus specimens(n=8)	50 (45.2–54.8)	100 (100–100)	50 (45.2–54.8)	100 (100–100)

a: Composite reference standard

b: Confidence interval

leading to a significant increase in pro-inflammatory cytokines such as interferon gamma (IFN-γ) and interleukin-6 (IL-6), and the production of anti-inflammatory cytokines such as interleukin-10 (IL-10) [11]. As shown in Fig. S3, case 56 warranted our vigilant attention. This patient had a positive result on TNPseq, with serum levels of IL-6, IFN-γ, and IL-10 that were different from those of non-BJTB patients. Initially, we suspected that this discrepancy was due to a false positive from the TNPseq assay. However, upon further analysis of the patient’s serologic markers, we observed that the levels of IL-6, IFN-γ, and IL-10 were significantly higher than those of non-tuberculous patients but lower than those of TB patients. This suggested that the patient might be a latent TB carrier with a compromised immune system. A previous study reported a similar case of an 85-year-old Caucasian woman with the same characteristics on initial admission [20]. Inadequate treatment due to subtle TB features led to two years of ineffective therapy, resulting in irreversible joint damage. This patient, case 56, will be followed in subsequent experiments. However, these findings are based on our experiment and are limited by the small sample size. In the future, we will expand our study to include more patients with positive TNPseq results but negative results from other tests. We will observe the development of these patients in subsequent disease progression, such as the possibility of latent tuberculosis. These observations will further clarify that TNPseq results are more predictive and accurate, providing a more reliable reference for clinical diagnosis.

This study demonstrated that TNPseq has a higher sensitivity and specificity for the detection of BJTB. Notably, the detection capability of the assay was significantly improved when pus specimens were used, which is consistent with the results reported by Kay et al. [21]. These results suggest that pus may be a preferred specimen type for the diagnosis of BJTB. Because this exploratory study is at an early stage, future research should include a variety of specimen types, including saliva, alveolar lavage fluid, puncture fluid, and transbronchial lung biopsies, to comprehensively evaluate and compare the performance of TNPseq with conventional culture methods [22]. Surprisingly, when puncture fluid and pus samples were

combined in TNPseq, the specificity remained at 100%, but the sensitivity dropped to 50%. This finding may be primarily due to the limited sample size rather than overly stringent diagnostic criteria applied to the mixed samples.

Nevertheless, these cases remain a subject for future discussion. In particular, a peripheral blood sample was negative in all diagnostic assays. We see this as a promising avenue for TNPseq, as the detection limit of nanopore technology continues to decrease. One investigate found that pathogen-derived DNA fragments are produced in response to infection and present in the bloodstream, providing ample opportunity for pathogen detection in blood samples, thereby eliminating the need for tissue or purulent fluid [16, 23]. This approach would greatly benefit patients by sparing them the physical discomfort associated with invasive sampling procedures [3].

Seventy pathogens were identified by ranking them based on the proportion of positive TNPseq results, demonstrating TNPseq’s ability to detect slow-growing pathogens. This ability mitigates the challenges of identifying non-tuberculous mycobacteria (NTM) and prevents NTM from confounding TB test results. *Candida albicans* and *Aspergillus* species were the most common fungi detected. As shown in a previous study, patients with aspergillosis were diagnosed and treated promptly, which significantly shortened their treatment duration [24]. Therefore, TNPseq could help clinicians better understand patients’ infections and promptly adjust their treatment strategies to minimize treatment-related discomfort. The unique strength of TNPseq lies not only in providing a definitive positive or negative result, but also in providing specific sequence information for pathogen detection, thereby providing valuable insights for clinical diagnosis [14]. Furthermore, by continuously analyzing and collating data on pathogenic bacteria and their incidence rates, potential novel pathogens associated with infections can be identified. This allows for targeted modifications of the clinical panel, further enhancing the benefits of TNPseq. However, due to the limited number of exploratory samples, this area remains a focus for future research and development.

Clinical treatment decisions were based on diagnostic findings, with broad-spectrum antibiotics being a common intervention. However, this approach was not universally effective. Failure to promptly identify and treat infections caused by multiple pathogens resulted in a significant treatment burden for patients [5, 12, 21]. For example, bacterial-fungal infections often lead to pulmonary fibrosis, respiratory failure, and in some cases, death [25]. TNPseq enables early and accurate diagnosis of pathogens, including slow-growing, bacterial-fungal and viral infections, providing a strong basis for clinical management and improving the prognosis of tuberculosis patients [24, 25]. The emergence of drug resistance genes poses a significant challenge to global tuberculosis control [26, 27]. Prior studies have utilized nanopore sequencing to identify resistance genes in various microbes [28, 29]. The introduction of TNPseq testing has the potential to reduce the overall duration of medical care and the risk of disabling complications caused by M.tb [30]. Cost and turnaround time are key considerations in tuberculosis diagnostics. In China, the cost of TNPseq testing per specimen is approximately \$65.7 and the turnaround time is only 14.5 h, as shown in Table S4. In comparison, T-SPOT testing ranges from \$73.05 to \$100, Xpert is \$70, and culture is \$30 [31]. TNPseq provides results within one day, while T-SPOT requires approximately 12 h [32]. Studies have shown that TNPseq can detect drug resistance genes or identify mixed pathogens, thereby significantly influencing the clinical management of TB patients and providing cost-effectiveness benefits [30]. In the course of subsequent treatment, we will continue to monitor patients' responses to treatment and provide a comprehensive evaluation of TNPseq for microbial and antimicrobial resistance.

## Conclusions

This study used TNPseq on 163 suspected BJTB specimens and demonstrated the potential of TNPseq-based assays as promising diagnostic tools for BJTB. TNPseq demonstrated higher sensitivity and specificity compared to T-SPOT and Xpert. In addition, TNPseq facilitated pathogen discovery, classification of mixed infections, and drug resistance analysis, providing valuable insights for clinical infection management. However, current research is limited by the relatively small sample size. Future studies will also require extensive analysis of medical records to further validate these findings.

## Limitations of this study

Our study has several limitations. First, as a retrospective, single-center study with a limited sample size, there may be an inherent bias. We speculate that this could be mitigated by including more clinical samples in future studies. Second, we did not discuss in detail the economic

burden of TNPseq for rural or urban TB treatment settings. In resource-limited settings, TNPseq may not be widely applicable. As sequencing technology advances, we anticipate that the cost of TNPseq will continue to decrease, thereby enabling its use in a broader range of clinical settings.

## Abbreviations

BJTB	Bone and joint tuberculosis
TNPseq	Targeted nanopore sequencing
M.tb	Mycobacterium tuberculosis
TB	Tuberculosis
PCR	Polymerase chain reaction
T-SPOT	T-lymphocyte spot
NGS	Next-generation sequencing
CRS	Composite reference standard
CT	Computed tomography
MRI	Magnetic resonance imaging
PPV	Positive predictive value
NPV	Negative predictive value
SD	Standard deviation
IQR	Interquartile range
PLR	Positive likelihood ratio
NLR	Negative likelihood ratio
AUC	Area under curve
NTM	Non-tuberculous mycobacteria

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12941-024-00733-z>.

Supplementary Material 1

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## Author contributions

Y.D and Q.R contributed to the study design. F.Z, G.L and T.L collected the data and carried out the laboratory testing. Z.S and Y.C helped with the bioinformatics analysis. J.W and Q.W occupied in initial manuscript drafting and revisions, H.G and Q.Z calibrated the data analysis and finally prepared the manuscript. All the authors read and approved the final manuscript.

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## Data availability

No datasets were generated or analysed during the current study.

## Declarations

### Ethical approval and consent to participate

This study protocol was conducted in accordance with the principles of Declaration of Helsinki. The local ethics committee approved the study protocol in Jinan, Shandong Province (No. GWLCZXC-AF-03-1-1), and all patients gave written informed consent to participate.

### Consent for publication

Not applicable.

## Competing interests

The authors declare no competing interests.

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