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ORIGINAL ARTICLE

The Value of Single-Molecule Nanopore DNA Sequencing in the Clinical Diagnosis of Suspected Tuberculosis Patients

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SUMMARY

Background: **Early diagnosis of *Mycobacterium tuberculosis* (MTB) infection is of great significance for the clinical management of tuberculosis (TB). We first explored the efficacy of single-molecule nanopore DNA sequencing in the early diagnosis of suspected TB patients and analyzed the advantages in differentiating and diagnosing MTB and non-*tuberculous Mycobacteria* (NTM).**
Methods: **In this cohort study, we reviewed the clinical data of suspected TB patients admitted from December 1, 2021, through April 15, 2022. All patients underwent 3 - 6 times acid-fast bacilli smear examinations of sputum, all of which were negative. To make a definitive diagnosis, we extracted specimens from the patients and performed specimen culture, Xpert MTB/Rif assay, and single-molecule nanopore DNA sequencing. The efficacy of different diagnostic methods in diagnosing suspected TB patients was compared using "Diagnostic Criteria for Pulmonary Tuberculosis" (WS288-2017) as the gold standard.**
Results: **Among the 25 patients, 15 were infected with MTB, 5 were infected with NTM, 1 had mixed MTB and NTM infection, and 4 were negative. The accuracy of single-molecule nanopore DNA sequencing in diagnosing mycobacterial infection (MTB + NTM) was 92.0%, with a sensitivity of 90.5% and a specificity of 100%; the accuracy of diagnosing MTB infection was also 92.0%, with a sensitivity of 87.5% and a specificity of 100%. Single-molecule nanopore DNA sequencing showed an accuracy of 100% in differentiating MTB and NTM. However, the diagnostic accuracy and sensitivity of specimen culture and Xpert MTB/Rif assay were relatively low (≤ 52%) compared to "specimen culture + Xpert MTB/Rif assay". The diagnostic efficacy of single-molecule nanopore DNA sequencing was not affected by the source of tissue samples, while specimen culture and Xpert MTB/Rif assay could not diagnose mycobacterial infection using extrapulmonary specimens.**
Conclusions: **As a third-generation sequencing technology, single-molecule nanopore DNA sequencing has significant application value in diagnosing suspected TB patients. Compared to traditional diagnostic methods, such as specimen culture and Xpert MTB/Rif assay, single-molecule nanopore DNA sequencing exhibits high diagnostic efficacy, low error rate, and convenient detection.**
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KEYWORDS

pulmonary tuberculosis, single-molecule nanopore DNA sequencing, mycobacterium tuberculosis, third generation sequencing, laboratory

INTRODUCTION

Tuberculosis (TB) has been among the top 10 causes of death worldwide for a long time [1]. Since 2007, TB has become the leading cause of death among all infec-

tious diseases, surpassing even human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) [2]. Approximately 2 billion people worldwide are infected with *Mycobacterium tuberculosis* (MTB), accounting for nearly one quarter of the global population [3]. Among them, about 5 - 10% of the infected individuals will progress to active tuberculosis. People infected with MTB who have HIV/AIDS, as well as those with risk factors such as diabetes and malnutrition, are at a higher risk of developing active tuberculosis [4]. Pulmonary tuberculosis, caused by MTB infection, is a common respiratory infectious disease. Due to its transmission through respiratory droplets, pulmonary tuberculosis is the most common type of TB. Although TB patients often achieve good prognosis after receiving clinical treatment, elderly patients and those with underlying diseases often require longer and more diverse treatment approaches and have difficulty achieving good prognosis [5]. Furthermore, with the spread of TB, an increasing number of clinical samples are testing positive for drug-resistant MTB, especially with the alarming situation of multidrug-resistant *tuberculosis* (MDR-TB) in recent years [6,7]. The WHO's global tuberculosis report for 2021 [8] has shown that the COVID-19 pandemic has reversed the progress made in providing basic tuberculosis care and reducing the burden of tuberculosis over the years. Due to the impact of the COVID-19 pandemic, global targets for tuberculosis have deviated, resulting in decreased expenditures for tuberculosis diagnosis, treatment, and prevention services, leading to an increase in tuberculosis-related deaths and a slowdown in the decline in incidence rates. The estimated number of deaths in 2020 has returned to the level of 2017, and the largest impact on incidence rates is projected to occur in 2022. This indicates that TB remains a highly infectious global public health problem. Therefore, early diagnosis of pulmonary tuberculosis patients and proper treatment are particularly important [4].

The key step in controlling the transmission of tuberculosis is early control of the infection source, and early diagnosis is also of great significance for patient treatment. Currently, many countries still rely on bacteriological examination methods, such as smear microscopy, for the diagnosis of MTB. However, smear microscopy has a low positive rate, requires a long time (2 - 6 weeks) to obtain positive culture, and has high technical requirements, making it unable to meet the clinical diagnostic and treatment needs [9]. Xpert MTB/Rif assay is a polymerase chain reaction (PCR)-based technology that can specifically detect specific sequences in the pathogen's genome, thus identifying the type and quantity of the pathogen, and it can complete the testing in a short time [10]. However, the sensitivity and specificity of Xpert MTB/Rif assay are still not ideal. With the continuous development of sequencing technology, there have been significant improvements in throughput and cost. Third-generation sequencing technologies, represented by single-molecule nanopore DNA sequencing, have made significant contributions in the fields of medicine and life sciences, with advantages such as ultra-long read lengths, real-time detection, and direct detection of base methylation modifications [11]. However, there is currently a lack of analysis on its diagnostic value in clinical suspected TB patients. This study systematically analyzed the detection efficacy of single-molecule nanopore DNA sequencing for suspected TB patients and analyzed its clinical application advantages.

MATERIALS AND METHODS

Database

In this cohort study, a total of 25 suspected TB patients treated between December 1, 2021, and April 15, 2022, were selected as the study subjects. Inclusion criteria: 1) patients with symptoms such as cough, sputum production, fever, and chest tightness; 2) patients who underwent 3 - 6 times acid-fast bacilli smear examinations of sputum specimens, all of which were negative; and 3) patients who completed specimen culture, Xpert MTB/Rif assay, and single-molecule nanopore DNA sequencing during the same period. Exclusion criteria: 1) patients with unclear clinical diagnosis; 2) patients with a history of TB; and 3) patients with missing clinical data. Among the 25 patients, there were 19 males and 6 females, with an age range of 23 to 71 years and an average age of 57 ± 19 years. The examined samples included bronchoalveolar lavage fluid (BALF) in 19 cases, pleural fluid in 2 cases, sputum in 2 cases, urine in 1 case, and intra-abdominal purulent fluid in 1 case. Supplementary Table S1 shows the detailed information of the patients.

Ethical approval and consent to participate

This study complies with the ethical principles of the Declaration of Helsinki. This study has obtained ethical approval from the Medical Research Ethics Committee of Anhui Provincial Chest Hospital (KJ2023-044). During the process of collecting research data, we have obtained informed consent from the patients, so the research subjects were aware of the research content and participated voluntarily.

Diagnostic criteria

The diagnostic criteria for pulmonary tuberculosis in "Diagnostic Criteria for Pulmonary Tuberculosis" (WS 288-2017) [12] are: confirmed by pathogen or pathological evidence and positive for MTB nucleic acid detection by molecular biology. According to the "Diagnostic Criteria for Pulmonary Tuberculosis" (WS288-2017), the final clinical diagnostic criteria in this study are: combination of clinical symptoms and signs, chest imaging examination, positive tuberculosis immunological tests, and effective response to standard anti-tuberculosis treatment, while excluding other diseases.

Specimen collection

BALF: The procedure was performed by using an Olympus 290 electronic bronchoscope (Japan). The affected lung segments were rinsed with physiological saline solution 2 - 3 times. Sputum: deep sputum was collected. Urine: midstream urine was collected. Samples from the pleural and abdominal cavities were obtained through sterile puncture procedures.

MTB detection methods

Specimen culture and drug susceptibility testing [13]: after standard processing of each specimen, it was inoculated into BACTEC MGIT 960 liquid culture medium, and the culture tube was placed in an incubator for incubation. The results were automatically reported by the instrument. The drug susceptibility test was performed by using the YK-909 Mycobacterium tuberculosis microplate reader (China).

Xpert MTB/Rif assay [14]: Xpert MTB/Rif was operated according to the instructions for use of the real-time fluorescence quantitative nucleic acid amplification detection system produced by Cepheid (USA).

Single-molecule nanopore DNA sequencing [15]: More than 5 mL of specimens, such as by bronchoalveolar lavage fluid (BALF) and sputum, were sent to the testing center for detection, and the new generation of nanoscale single-molecule sequencing technology was used for microbial classification identification [15]. The sequencing results were matched with the comparative microbial database, including *Mycobacterium tuberculosis* complex, 168 types of non-tuberculous mycobacteria (NTM), 10 common microbial resistance, 16 types of first and second-line *tuberculosis* drug resistance genes, 5 types of non-tuberculosis drug resistance genes, and more than 12,000 types of bacteria, fungi, and viruses.

Statistical analysis

SPSS 23.0 (IBM, USA) statistical software was used for data analysis. Count data were expressed as "n (%)" and analyzed using Pearson's chi-squared test with a significance level of $\alpha = 0.05$ (two-tailed). Non-normally distributed measurement data were expressed as median (interquartile range) "M (QR)" and compared between groups using Mann-Whitney U test. Normally distributed measurement data were expressed as "$x \pm s$" and compared within groups using paired sample *t*-test. Repeated measurement data were analyzed using repeated measures analysis of variance, and post hoc comparisons were performed using the Student-Newman-Keuls test. A significance level of $\alpha = 0.05$ (two-tailed) was selected. $p < 0.05$ indicated a statistically significant difference.

RESULTS

Laboratory detection results

The laboratory data of 25 patients were included in the study (Supplementary Table S1). Among the 21 suspected TB patients, 15 were diagnosed with MTB infection and 5 were diagnosed with NTM infection, with 1 case of mixed MTB and NTM infection. Single-molecule nanopore DNA sequencing results indicated that among the 5 NTM infected patients, 3 had *Mycobacterium abscessus* infection (Nos. 8, 9, and 19), and 2 had *Mycobacterium intracellulare* infection (Nos. 14 and 16). The patients with mixed infection (MTB + NTM) were simultaneously infected with MTB, *Mycobacterium abscessus*, and *Mycobacterium chelonae* (No. 2). Drug susceptibility testing results showed that 6 drug-resistant tuberculosis patients were all infected with MTB, including 1 case resistant to all 6 anti-tuberculosis drugs (rifampicin, isoniazid, ethambutol, streptomycin, pyrazinamide, and fluoroquinolones) (No. 12), 1 case resistant to rifampicin and streptomycin (No. 20), and the remaining 4 cases resistant to a single anti-tuberculosis drug (No. 3 resistant to isoniazid, No. 10 resistant to streptomycin, and Nos. 11 and 17 resistant to rifampicin).

Efficacy of different detection methods in diagnosis of suspected TB

By using the WHO-acceptable clinical diagnosis as the "gold standard", we analyzed the value of different detection methods in the diagnosis of suspected TB patients. "specimen culture + Xpert MTB/Rif assay" refers to the combined results of specimen culture and Xpert MTB/Rif assay. When either the specimen culture or Xpert test result is positive, the diagnosis of "specimen culture + Xpert MTB/Rif assay" is considered positive. When both the specimen culture and Xpert MTB/Rif assay results are negative, the diagnosis of "specimen culture + Xpert MTB/Rif assay" is considered negative. Table 1 shows the value of different detection methods in the diagnosis of mycobacterial infection (MTB + NTM). All four detection methods displayed excellent specificity (100%). In terms of sensitivity, single-molecule nanopore DNA sequencing showed significant advantages compared to specimen culture, Xpert MTB/Rif assay, and "specimen culture + Xpert MTB/Rif assay" (92.0% vs. 40.0%, 32.0%, and 44.0%). The diagnostic accuracy of single-molecule nanopore DNA sequencing was as high as 92.0%, while the other three detection methods had diagnostic accuracies below 50%.

Table 2 shows the value of different detection methods in the diagnosis of MTB. Similar to their performance in the diagnosis of mycobacterial infection, single-molecule nanopore DNA sequencing demonstrated significant advantages in the diagnosis of MTB (accuracy: 92%, sensitivity: 87.5%, and specificity: 100%). Compared to specimen culture and Xpert MTB/Rif assay, the combination of " specimen culture + Xpert MTB/Rif assay" did not improve the diagnostic accuracy.

Table 1. Efficacy of different detection methods for diagnosing mycobacterial infection (MTB + NTM).

Detection methods	Accuracy	Sensitivity	Specificity	PPV	NPV
Single-molecule nanopore DNA sequencing	92.0%	90.48%	100.0%	100.0%	66.67%
Specimen culture	40.0%	28.57%	100.0%	100.0%	21.05%
Xpert MTB/Rif	32.0%	19.05%	100.0%	100.0%	19.05%
Specimen culture + Xpert MTB/Rif	44.0%	33.33%	100.0%	100.0%	22.22%

PPV - positive predictive value, NPV - negative predictive value.

Table 2. Efficacy of different detection methods for diagnosing MTB.

Detection methods	Accuracy	Sensitivity	Specificity	PPV	NPV
Single-molecule nanopore DNA sequencing	92.0%	87.5%	100.0%	100.0%	81.82%
Specimen culture	44.0%	25.0%	77.78%	66.67%	36.84%
Xpert MTB/Rif	52.0%	25.0%	100.0%	100.0%	42.86%
Specimen culture + Xpert MTB/Rif	48.0%	31.25%	77.78%	71.43%	38.89%

PPV - positive predictive value, NPV - negative predictive value.

After excluding four cases of negative mycobacterium and one case of mixed MTB and NTM infection, 20 samples were used to analyze the value of single-molecule nanopore DNA sequencing in the differentiation diagnosis of MTB and NTM. The accuracy of single-molecule nanopore DNA sequencing in the differentiation diagnosis of MTB and NTM was 100%.

Diagnostic efficacy of different detection methods in intra-pulmonary and extra-pulmonary specimens

The type of specimen tested may have an impact on the detection results. We classified the specimens into intra-pulmonary specimens (BALF and sputum) and extra-pulmonary specimens (pleural effusion, ascites, and urine). As shown in Supplementary Table S2, in both intra-pulmonary and extra-pulmonary specimens, single-molecule nanopore DNA sequencing has a very high diagnostic sensitivity [intra-pulmonary specimens: 88.24% (15/17) for mycobacterial infection (MTB + NTM) and 91.67% (11/12) for MTB; extra-pulmonary specimens: 100% (4/4) for mycobacterial infection (MTB + NTM) and 100% (3/3) for MTB]. Especially in extra-pulmonary specimens, the diagnostic results of single-molecule nanopore DNA sequencing is consistent with clinical diagnoses. When it comes to specimen culture and Xpert MTB/Rif assay, they do not have diagnostic value in extra-pulmonary specimens (sensitivity: 0%, specificity: 0%).

DISCUSSION

Currently, traditional cultivation methods are still widely used for the diagnosis of clinical tuberculosis. However, these methods are time-consuming, specimens are prone to contamination, and they can lead to missed diagnosis and transmission of drug-resistant patients, failing to meet the requirements for rapid clinical diagnosis of tuberculosis. Acid-fast bacilli examination using sputum smears is a rapid and cost-effective testing method, but it has lower sensitivity and specificity and cannot be used for drug susceptibility testing; it can only serve as the preferred method for preliminary clinical screening [9]. With the continuous development of molecular biology techniques, various molecular technologies have been applied to the diagnosis of tuberculosis. Gene chip technology is based on gene mutations of rpoB/katG/inhA, which can rapidly detect isolates, report specific mutation types, and detect rifampicin and isoniazid-resistant mycobacteria in samples. However, it has complex operation and poor repeatability [16]. Melting curve technology uses multi-color probe melting curve method to rapidly detect common drug-resistant determination regions of Mycobacterium tuberculosis to rifampicin, isoniazid, and quinolone. Tuberculosis identification and drug resistance need to be tested separately, with a higher false positive rate [17]. The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) system is used for the analysis, which can detect specific mutation sites of first-line and second-line drugs such as rifampicin, iso-

niazid, and quinolones. However, it cannot cover all drug-resistant gene determination regions, requires high bacterial content in samples, and may have a high false negative rate [18]. Xpert MTB/Rif technology is an optimized fluorescent quantitative PCR technique, which can rapidly and accurately detect the specific sequence of the rpoB gene and the 81 bp core interval determining rifampicin resistance in Mycobacterium tuberculosis. The detection time is shortened to 1 day. However, the disadvantage is that it can only detect rifampicin resistance and does not report specific mutation types. Synonymous mutations can lead to false positive results [10,19]. All of the abovementioned techniques can only detect MTB and cannot detect NTM and other cases of mixed infections. Therefore, more accurate and convenient detection methods are urgently needed in clinical practice.

With the rapid development of nucleic acid sequencing technology, it has been widely applied in various fields such as molecular biology, evolutionary biology, metagenomics, medicine, and forensic science as an efficient, fast, and low-cost research and detection method. However, due to the limitations of sequencing technology itself, the first-generation sequencing technology is not suitable for large-scale sequencing of multiple samples, and the second-generation sequencing technology is limited in application due to its short read length and high GC bias [20]. The third-generation sequencing technologies, represented by single-molecule nanopore DNA sequencing from Oxford Nanopore Technologies (ONT) and single molecule real-time sequencing (SMRT) from Pacific Biosciences (PacBio), have been increasingly used in genome assembly, epigenetic markers, transcriptomics, and metagenomics due to their high throughput, long read length, and direct detection of base methylation modifications [11,21]. Compared to SMRT technology, single-molecule nanopore sequencing technology not only has advantages in terms of read length (up to 2 Mb), but also has a smaller device size, making it easier to carry. Therefore, it has significant advantages in real-time sample detection in extreme and relatively backward experimental conditions in epidemic areas [22]. Since 2017, the nanopore sequencing platform has mainly used two sequencing strategies, $1D^1$ and $1D^2$. The principle of the $1D^1$ sequencing strategy is as follows: the genomic DNA or cDNA molecules are assisted by adapters to reach near the nanopore, and the double-stranded DNA molecules are unfolded into single strands under the action of helicase and passed through the pore protein. The sensor detects the differences in current caused by different nucleotides and converts them into electrical signals. Finally, based on the spectrum of changes in the electrical signal, the applied pattern recognition algorithm determines the type of base. Unlike the $1D^1$ sequencing strategy, the $1D^2$ sequencing strategy adds a special adapter to the two DNA molecules during library preparation, allowing the complementary strand to attach to the membrane while the template strand is being read. Shortly after the first strand leaves the nanopore, there is a certain probability that the complementary strand will be sequenced, and the data from the two strands will correct each other, helping to improve the accuracy of sequencing [22,23]. However, there have been no systematic reports on the application of single-molecule nanopore DNA sequencing in diagnosing suspected TB patients in clinical practice.

Specimen culture and Xpert MTB/Rif assay are commonly-used clinical methods for detecting MTB. We compared their efficacy in diagnosing TB patients with that of single-molecule nanopore DNA sequencing. Furthermore, we combined the results of specimen culture and Xpert MTB/Rif assay in a "sequential" manner as a diagnostic method, referred to as "specimen culture + Xpert MTB/Rif assay". By using the final clinical diagnosis as the "gold standard," the diagnostic accuracy of single-molecule nanopore DNA sequencing reached 92.0%, while the diagnostic accuracy of other detection methods was less than 50%. Considering that only single-molecule nanopore DNA sequencing can detect NTM, we separately analyzed the performance of the four diagnostic methods for MTB diagnosis. Single-molecule nanopore DNA sequencing (accuracy: 92%, sensitivity: 87.5%, specificity: 100%) still demonstrated significant advantages in MTB diagnosis. Moreover, compared to specimen culture and Xpert MTB/Rif assay, "specimen culture + Xpert MTB/Rif assay" did not improve the diagnostic accuracy. This also suggests that clinical attention should be more focused on improving detection techniques, as relying solely on combining multiple approaches may not necessarily enhance diagnostic efficacy. NTM refers to non-tuberculous mycobacteria other than MTB and leprosy bacilli. Due to the similarities in clinical manifestations and imaging examinations with MTB, NTM can sometimes cause mixed infections with MTB, leading to misdiagnosis, missed diagnosis, and treatment delays for patients. Therefore, effective differential diagnosis is of great significance for patient treatment. The study results showed that single-molecule nanopore DNA sequencing achieved an accuracy of 100% in MTB and NTM differentiation diagnosis. It is worth noting that the diagnostic efficacy of the sequencing technology in MTB in this study was significantly better than previous reports [24,25]. We analyzed the reasons, including: 1) the use of single-molecule nanopore DNA sequencing, which has the advantages of high throughput, ultra-long read length, and the ability to directly detect base methylation modifications; 2) the single-molecule nanopore DNA sequencing analysis in this study was based on the $1D^2$ sequencing strategy, which effectively improved the sequencing accuracy; and 3) MTB has a thick cell wall and is difficult to disrupt, while we adopted a dual enzymatic and mechanical grinding efficient disruption technique, which effectively improved the quality of nucleic acid extraction from the specimens. In addition, we also analyzed the differences in diagnostic efficacy of different detection methods between intra-pulmonary

tissue samples and extra-pulmonary tissue samples. Chen [26] et al.'s study showed that the overall sensitivity of high-throughput sequencing was 49.6%, with the highest sensitivity in biopsy lung tissue (88.9%), followed by BALF (55.0%), and then pleural effusion (50.0%). Shi [27] et al.'s study also indicated that high-throughput sequencing exhibited better diagnostic capability in intra-pulmonary tuberculosis than extra-pulmonary tuberculosis. In this study cohort, only four suspected TB patients had extra-pulmonary specimens collected. Specimen culture and Xpert MTB/Rif assay were negative, which may be due to the low concentration of mycobacteria in pleuroperitoneal fluid and urine samples, making it difficult for conventional methods to detect them. The single-molecule nanopore DNA sequencing analysis used in this study provided accurate diagnosis, possibly because this technology allows for low starting DNA library preparation (DNA concentration as low as 0.1 ng - 1 ng/μL for library preparation and sequencing) [28], greatly improving the detection sensitivity of extra-pulmonary specimens.

This study has some limitations: 1) it is a retrospective cohort study with a small sample size, which may have certain biases; 2) only four suspected TB patients with retained extra-pulmonary specimens were included in the study, making it impossible to analyze the diagnostic efficacy; and 3) the patients in this study did not undergo second-generation sequencing or first-generation sequencing, and therefore, the comparative diagnostic efficacy of single-molecule nanopore DNA sequencing for suspected TB patients could not be analyzed. In the future, we will further analyze the application advantages of single-molecule nanopore DNA sequencing in TB diagnosis and other clinical diseases through prospective, large-sample cohort studies.

CONCLUSION

Single-molecule nanopore DNA sequencing technology can rapidly and accurately identify the types of pathogenic bacteria, especially for mixed infections and NTM infections. It can also perform comprehensive detection of multiple drug-resistant genes at all loci. The application value of single-molecule nanopore DNA sequencing technology is significantly higher than that of traditional specimen culture and Xpert MTB/Rif assay in the clinical diagnosis of suspected TB patients.

Declaration of Interest:

This submission constitutes original work that has not been published previously and is not being reviewed or considered for publication elsewhere. The authors declare that there are no conflicts of interest associated with this manuscript. The submitting author confirms that all authors have met the authorship criteria and that the manuscript has been read and approved for submission by all authors named.

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