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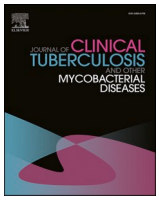

Comparative analysis of five etiological detecting techniques for the positive rates in the diagnosis of tuberculous granuloma

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ABSTRACT

Purpose: To examine the relationship between the positive rate and types of necrosis in pathological examinations of tuberculosis granulomas with necrosis, to improve the detection rate of positive cases.
Methods: Specimens from 381 patients were collected in Wuhan Pulmonary Hospital from Jan 2022 to Feb 2023. The samples were examined using various methods such as AFB smear microscopy, mycobacterial culture, PCR, SAT-TB, and X-pert MTB/RIF rapid molecular detection.
Result: There were 3 types of necrosis. Including 270 cases of caseous necrosis, 30 cases of coagulation necrosis, and 76 cases of an abscess. Five cases were non-necrotizing granulomas.
In the pathological specimen testing for tuberculosis, five detection techniques were used and their positive rates detected in descending order were X-pert, TBDNA, SAT-TB, tuberculosis culture, AFB. Comparison between different examinations in the group: X-pert had the highest positive rate in each group, and it was significantly higher than TBDNA ($P < 0.01$) in caseous necrosis specimens. Compared with the same examination between the groups, the detection rates of X-pert and TBDNA in abscess and caseous necrosis specimens were significantly higher than in coagulation necrosis specimens ($P < 0.01$).
Conclusion: The positive rates of the five etiological detection techniques in tuberculous granuloma with different types of necrosis were quite different. The specimens of caseous necrosis or abscess could be selected for detection, and X-pert had the highest positive rate.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (MTB) infection [1] and is the main infectious cause of global morbidity and mortality [2]. The incidence of extrapulmonary tuberculosis is increasing year by year [3], and tuberculosis-related surgery is not rare in all kinds of hospitals, but its diagnosis is still difficult [4].

Due to insufficient emphasis and inadequate diagnostic methods, the clinical diagnostic rate of extrapulmonary tuberculosis is not high, and its cure rate has always been lower than that of pulmonary tuberculosis, which poses a challenge to the global control of tuberculosis. As most surgical specimens are difficult to repeat, and granulomatous inflammation is the most common pathological change in tuberculosis, which lacks specificity [5], therefore, how to improve the pathogenic detection rate of postoperative tuberculosis specimens, especially how to detect drug-resistant tuberculosis as early as possible, is a question worthy of clinical and pathological attention. This study explores the relationship between the positive rate (sensitivity) of tuberculosis pathogens in pathological specimens and the type of pathological changes, and how to improve the pathological positive rate through precision sampling, using clinical diagnosis as the gold standard and surgical cases as the research object.

2. Materials and methods

2.1. Ethics approval

The ethics committee of Wuhan Pulmonary Hospital (2022–61) reviewed and approved the study protocol. All participants gave written informed consents before enrollment. Patient records and information were de-identified prior to analysis.

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2.2. Process flow at the hospital

Wuhan Pulmonary Hospital receives TB patients from central China, and counties and surrounding areas. A study was conducted on 381 patients with extrapulmonary TB and granulomatous inflammation at the hospital from January 2022 to February 2023.

2.3. Study population and data collection

This study comprised of 198 males and 183 females, spanned ages from 3 months to 81 years, with an average age of 34.77 ± 18.60 years. The patients, were diagnosed with TB after undergoing tissue sample testing from various sites.

2.4. Mycobacterium culture

In this study, pellets from each sample were subjected to treatment and subsequently inoculated into Middlebrook 7H10 solid slopes and liquid culture broth mycobacterial growth indicator tubes (MGIT) to facilitate growth. The cultures were maintained under optimal conditions, i.e., at a temperature of 37 °C for up to 6 weeks and 8 weeks for the liquid and solid media, respectively [6]. Positive identification of mycobacterial isolates was confirmed using Ziehl-Neelsen staining, while biochemical tests, carried out using molecular probes (Accuprobe) were utilized to observe colony morphology and/or nucleic acid hybridization for species identification. Biochemical identification tests for niacin accumulation and nitrate reduction were performed on Lowenstein-Jensen solid medium, with inoculation from 7H10 or MGIT isolates that showed positive mycobacterial growth. The results obtained included both negative and positive findings, with positive results primarily indicating the presence of *Mycobacterium tuberculosis* complex (MTBC) and *non-tuberculous mycobacteria* (NTM).

2.5. AFB smear microscopy

The current study employed the N-acetyl-l-cysteine–NaOH (NALC–NaOH) technique to process specimens for both digestion and decontamination purposes [7]. Following centrifugation at 3200 × g for 20 min, the specimen was concentrated, and the precipitate was reconstituted with 0.067 Msterile phosphate buffer (pH 6.8). The processed deposits underwent microscopic examination via smear microscopy utilizing Auramine O fluorescent dye and Ziehl–Neelsen staining for confirmation [8]. The scoring of smear-positive specimens classified in accordance with the guidelines outlined by the US Centers for Disease Control and Prevention8 range from 1+ to 4+.

2.6. Gene Xpert MTB/RIF

The diagnostic assay Gene Xpert MTB/RIF was performed utilizing the GeneXpert Dx system [9]. Sediment samples that were degraded, decontaminated, and concentrated were suspended in a volume of 0.5 mL and transferred to a conical screw-cap tube. Subsequently, 1.5 mL of Xpert MTB/RIF sample reagent was introduced in the tube via a sterile pipette, and the tube was agitated 10–20 times. The sample was then incubated within the range of 20–30 °C for a total of 15 min, initiating the incubation phase sometime between 5 and 10 min. Following this step, the sample treated with the reagent was moved to the sample chamber of the Xpert MTB/RIF column using a sterile pipette and subsequently loaded into the GeneXpert Dx instrument system for processing.

2.7. Quantitative real-time PCR

Following the standard PCR protocol, the fluorescein-labeled TaqMan probe was mixed with template DNA, and underwent a thermal cycle of high-temperature denaturation, low-temperature renaturation, and temperature extension. Upon cleavage of the TaqMan probe that is complementary to the template DNA, fluorescein was freed and emitted fluorescence when excited by specific light sources [10]. As the number of cycles increased, the amplification of the target gene fragments occurred exponentially, which was detected in real-time by the corresponding changes in fluorescence signal intensity. The Taqman probe that is complementary to the template DNA is cut. Fluorescein is free in the reaction system and emits fluorescence under specific light excitation. As the number of cycles increases, the amplified target gene fragments increase exponentially, and the corresponding fluorescence signal intensity changes with amplification through real-time detection, Calculate the Cycle threshold and use several standard samples with known template concentrations as controls to get the copy number of the target gene in the test specimen.

2.8. SAT-Tb

The pretreatment of the samples involved adding 1.0 mL of the sample to a mixture of 2 mL of 4% NaOH, followed by thorough mixing with a vortex shaker, and leaving it to stand at room temperature for 15 min. Subsequently, 1.0 mL of the mixture was transferred to a 1.5 mL centrifuge tube and centrifuged at 13,000 revolutions per minute for 5 min. The resulting supernatant was discarded and the precipitate was mixed with 1.0 mL of sterile normal saline, followed by centrifugation at 12,000 revolutions per minute for 5 min [11]. For TB-RNA extraction, 50 μL of dilution was added to the precipitate and resuspended, and then sonicated for 15 min at 300 W. In the amplification test, 2 μL of treated sample supernatant was added to the.

TB-RNA reaction tube, followed by addition of 30 μL of amplification reaction solution. The reaction tube was placed on a dry-heat thermostat, with temperature maintained at 60 °C for 10 min, then at 42 °C for 5 min. Meanwhile, the enzyme solution was preheated at 42 °C for 10 min. Followed by immediate amplification by fluorescence quantitative PCR instrument using 42 μL of the reaction system, with FAM channel detection and amplification reaction carried out for 1 min at 42 °C, for a total of 40 cycles.

2.9. Pathological data collation

After undergoing a rigorous double-blind review process, the reports detailing the presence of “granulomatous inflammation” were analyzed for their description of pathological necrosis and subsequently categorized based on the type of necrosis observed. In cases where multiple types of necrosis were present, they were classified in order of suppurative inflammation/abscess, caseous necrosis, and coagulation necrosis.

2.10. Statistical methods

Data were entered into IBM SPSS Statics version 23.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA). The positive rate of each group was calculated, and the $\chi 2$ test was used for comparison between groups, $P < 0.05$ was considered statistically significant, and Chi-square segmentation was used for pairwise comparison.

3. Results

3.1. Granulomatous inflammation

Can be divided into two main categories according to the presence or absence of necrosis, namely, non-necrotizing granulomas and granulomas with necrosis. There were three types of caseous necrosis (70.87%, 270/381), abscess (19.94%, 76/381) and coagulation necrosis (7.87%, 30/381). As shown in Table 1, the positive rates of all tuberculosis etiology of the three types were in the same order from high to

low, which were X-PERT, TB-DNA detection, SAT-TB, tuberculosis culture, and smear acid-fast staining. Five cases of non-necrotizing lesions (4 cases of non-necrotizing epithelioid cell granulomatous inflammation and 1 case of non-necrotizing granulomatous inflammation) were negative for all types of tuberculosis.

3.2. Comparison between different tests within the group

The X- pert and TB- DNA positive rates for abscesses and caseous necrosis specimens were significantly higher than those of acid-fast staining, SAT-TB, and tuberculosis culture ($P < 0.01$). The Xv pert for caseous necrosis was higher than TB-DNA ($P < 0.01$). The positive rate for X pert in coagulation necrosis specimens was not significantly different from TB – DNA and SAT-TB, but was significantly higher than acid-fast staining and tuberculosis culture ($P < 0.01$). There was no statistically significant difference between acid-fast staining and tuberculosis culture within each group.

3.3. Comparison between different tests among groups

There was no statistically significant difference between acid-fast staining, SAT-TB, and tuberculosis culture. The positive rates of TB-DNA and X-pert in coagulation necrosis specimens were significantly lower than those of caseous necrosis and abscess specimens ($P < 0.01$), but there was no statistically significant difference between the latter two types of specimens.

4. Discussion

Recently, the health administrative department released the mandatory health industry standard “WS288-2017 Diagnosis of Pulmonary Tuberculosis”, which changes the confirmed diagnosis of pulmonary tuberculosis from one with positive microbiology results to one with positive pathogen results (including microbiology and molecular biology) or positive pathological results [12]. Molecular pathology has the advantage of precise sampling [13], but there is a lack of systematic reporting on how to conduct pathogen tests in tissue pathological specimens that are difficult to obtain repeatedly in a timely manner with high detection rates and can provide reference for early diagnosis and treatment of diseases, as well as how to improve the positive rate of molecular pathology by sampling. These issues should be given attention.

4.1. Characteristics and clinical significance of five testing techniques

Common tuberculosis pathogen detection projects in clinical practice include acid-fast staining, tuberculosis culture, TB- DNA, X- pert, SAT-TB, etc [14]. Each technique has its own characteristics. Except for acid-fast staining, the other tests can confirm tuberculosis with high specificity [15], but the sensitivity varies greatly depending on the test object and reference standard, and they each have their own value for clinical diagnosis and treatment [15,16].

4.1.1. Acid-fast staining

Direct microscopic examination after staining of bacteria, which is simple and cheap [17], is commonly used for formalin-fixed paraffin-embedded (FFPE) tissues. However, its sensitivity is the lowest among the five methods [15], and in today’s increasing incidence of non-tuberculous mycobacteria, positive results cannot be used as a basis for the diagnosis of tuberculosis [18], and its clinical significance is not as important as tuberculosis culture and molecular biology detection.

4.1.2. Tuberculosis culture

Cultivation of bacteria with biological activity in the specimen, which cannot be used for PPFE tissues.The identification of the bacterial type and drug sensitivity results based on its positive results can guide clinical drug use, and its role is still irreplaceable [19]. It has high specificity and is often used as the gold standard for tuberculosis diagnosis [20]. However, its positive rate is easily affected by factors such as anti-tuberculosis treatment time and culture medium [21], and its long cultivation cycle is its disadvantage. According to the clinical diagnosis as the reference standard, the sensitivity of tuberculosis culture in this study was only 17.11%, which is lower than the 41.9% of MGIT 960 cultivation of dead bone tissue specimens and far lower than the 88.62% of X-pert [22].

4.1.3. SAT-Tb

Using the specific 16S rRNA of *Mycobacterium tuberculosis* as the target for amplification, a positive result indicates the biological activity of *Mycobacterium tuberculosis* and can serve as one of the indicators for monitoring the effectiveness of pulmonary tuberculosis treatment [23]. Qiu C et al. tested 960 suspected tuberculosis patients with SAT-TB and found a sensitivity of 57.3% and a specificity of 92.5% [11]. This study showed that if the results of tuberculosis culture were used as a reference, the sensitivity of SAT-TB was 70. 18% (40/57), the specificity was 66.22% (196/296), the positive predictive value was 28.57% (40/140), and the negative predictive value was 92.02% (196/213). If the clinical diagnosis was used as the reference, the sensitivity of SAT-TB was 40. 11% (140/349), which was significantly lower than X-pert and TB-DNA in combined abscess and caseous necrosis samples, so its diagnostic value was not as good as those two methods. However, it can be used as a predictive indicator for negative tuberculosis culture. Due to its positive rate being easily affected by multiple factors and its activity being greatly reduced after formalin treatment, it is not recommended for use in FFPE tissue examination.

4.1.4. TB-DNa

Amplification of *Mycobacteriu tuberculosis*-specific DNA [24], with higher detection rates in FFPE specimens than acid-fast staining, positive results can diagnose tuberculosis, but have no direct reference value for treatment. Based on clinical diagnosis, the sensitivity of TB-DNA in this study was 77.63%, which is lower than the 96.67% in FFPE tissue specimens of pulmonary and pleural tuberculosis (with a specificity of 100%), suggesting that precise sampling may improve the positive rate.

4.1.5. Xpert

A fully automated nucleic acid amplification detection technology based on semi-quantitative real-time PCR technology [25]. It can detect the core interval (RRDR) of rifampin-resistant genes as the target gene [25,26]. Results can be obtained within 2 h, which is helpful for both diagnosis and treatment, and is endorsed by the WHO [27]. Literature reports its specificity ranges from 98.0% to 99.8%, but its sensitivity varies greatly (29.0% to 96.0%) depending on the source of the specimen and the reference standard used [26]. However, Xpert has higher sensitivity than traditional acid-fast staining of smears and tuberculosis culture [15] (except for pleural fluid specimens) and is widely used in the examination of clinical liquid specimens. This study shows that the sensitivity of Xpert using tuberculosis culture as the gold standard was 98.46% (64/65); when clinical diagnosis was used as the standard, Xpert sensitivity was significantly higher than acid-fast staining of smears, SAT-TB, and tuberculosis culture in both abscesses and caseous specimens, consistent with literature reports. In caseous necrotic specimens, Xpert sensitivity was 93.66%, significantly higher than TB-DNA’s 81.48%. Additionally, although Xpert does not distinguish bacterial activity, it can screen for rifampin resistance in the early stages [25] when the tuberculosis culture positive rate is significantly lower than Xpert (in this study, only 9 of the 30 patients screened for rifampin resistance by Xpert were tuberculosis culture-positive, and their drug sensitivity results all confirmed rifampin resistance, with a confirmation rate of 100%), which is conducive to the implementation of precision medicine. Like TB-DNA, X-pert examinations are also suitable for FFPE tissues that have been sliced and deparaffinized [28], and it has a broad

clinical application prospect.

4.2. The relationship between the positive rate of five pathogen detection technologies and specimen properties

According to literature reports, both TB-DNA and Xpert have high specificity, but there are significant differences in sensitivity [15], which may be related to the source and condition of the specimens. Research on the influence of specimen collection on the positive rate of various tests has shown that the positive rate of tuberculosis pathology specimens is related to the collection method, and Antonangelo L et al. suggest that pleural tissue is more suitable than pleural effusion as a sample for Xpert detection [29,30]. Zhang et al. found that Xpert had a detection rate close to 100% in pus, but a very low detection rate in cerebrospinal fluid and pleural effusion [31]. Although our study showed no statistically significant differences between the three groups of necrotic specimens and acid-fast staining, SAT-TB, and tuberculosis culture, we believe that the positive rates of TB-DNA and Xpert are related to specimen properties based on the following observations. (1) In the early stage of our analysis of pathological specimens obtained through different methods, we found that the X-PERT positive rate of the purulent discharge specimens can reach 100% (37/37), while this study showed that the detection rates of all kinds of non-necrotizing granulomas were zero, indicating that the presence of necrotic material in the specimen is a critical factor in determining the positive rate. (2) The positive rates of various tests for caseous necrosis were lower than those for cheese-like necrosis and abscess specimens, with TB-DNA and X-PERT being significantly more effective, while there was no statistically significant difference between cheese-like necrosis and abscess specimens, suggesting that the sampling objects should be cheese-like necrosis or abscesses. (3) Pathological specimens showing suppurative changes had X-PERT positive rates of 89.33% and TB-DNA positive rates of 85.53%, while both could reach 100% in abscess specimens, indicating that the positive rate could be improved if more accurate necrotic specimens are selected for testing under the microscope. Based on the above facts, we suggest the following principles for specimen collection: FFPE specimens should be selected from wax block tissues with cheese-like necrosis or suppurative necrotic material for sectioning, deparaffinization, and X-PERT or TB-DNA testing, fresh surgical specimens should leave as much necrotic material as possible under direct visualization, and puncture specimens should confirm the discovery of necrotic material before sending them for pathogen testing. Our study shows that cases with combined cheese-like necrosis and abscess pathological changes account for 90.81% of the total, and improving the collection of these two types of specimens could increase the detection rate of tuberculosis pathogens.

The difference between our study and previous ones is that, in addition to routine pathological detection, we first systematically proposed and verified the difference in pathogenic detection results in pathological examinations. Previous reports have proposed differences between two or three detection methods, but we systematically compared five detection techniques. At the same time, according to our results, the factors that affect the detection technique results are actually related to the source and conditions of the specimens. In this study, we first proposed that different surgical specimens or different detection techniques can be used to improve the detection of tuberculosis pathology in clinical work.

5. Conclusion

In summary, in order to improve the detection rate of tuberculosis molecular pathology, optimization should be made in both testing items and sampling objects. It is recommended to perform X-PERT testing on specimens with necrosis (especially cheese-like necrosis or abscess lesions). However, this study showed that the positive rate of specimens with coagulation necrosis was low, which may be related to improper handling of the specimens or insufficient selection of necrotic specimens under the naked eye. In addition, the sample size of this study varies greatly, and it is worth further research and verification whether this affects the relevant results.

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Ethics approval

The ethics committee of Wuhan Pulmonary Hospital (2022-61) reviewed and approved the study protocol. All participants gave written informed consents before enrollment. Patient records and information were de-identified prior to analysis.

All procedures in this study were carried out in accordance with relevant laws and institutional guidelines and were approved by an ethics committee. Informed consent was obtained from patients, and privacy rights were always observed.

CRediT authorship contribution statement

Qibin Liu: Writing – review & editing. **Feng Xu:** Conceptualization, Methodology, Software. **Qiliang Liu:** Data curation, Writing – original draft. **Xiaoyu Liu:** Visualization, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Qibin Liu reports financial support was provided by Wuhan Science and Technology Bureau.

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