Original Article
Application of GeneXpert MTB/RIF assay in the diagnosis of bacteriologically-negative pulmonary tuberculosis

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Abstract: Objective: We aimed to investigate the diagnostic performance of GeneXpert MTB/RIF assay in patients with bacteriologically-negative pulmonary tuberculosis (TB). Methods: A total of 125 suspected TB patients who underwent surgery in Beijing Chest Hospital were selected for the study and divided into the TB group (n=83, including 57 patients with bacteriologically-negative pulmonary TB) and the non-TB group (n=42) according to their clinical diagnosis results. The Ziehl-Neelsen staining, mycobacterium TB gene amplification test (TB-DNA), and GeneXpert MTB/RIF assay were performed on formalin-fixed paraffin-embedded (FFPE) tissue samples of the patients postoperatively. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of these methods for the detection of mycobacterium TB (MTB) were evaluated. Results: The sensitivities of the GeneXpert MTB/RIF, TB-DNA, and acid-fast smear (AFS) tests to detect MTB in patients with pulmonary TB were 71.1%, 81.9%, and 43.4%, respectively, and the specificities were 100%, 97.6%, and 97.6%, respectively. The sensitivities of the GeneXpert MTB/RIF, TB-DNA, and AFS testing methods in detecting MTB in patients with bacteriologically-negative pulmonary TB were 61.4%, 75.44%, and 35.09%, respectively, and the specificities were 61.4%, 75.5%, and 35.1%, respectively. In the detection of resistance to rifampicin in patients with bacteriologically-negative pulmonary TB, the sensitivity and specificity of Gene-Xpert MTB/RIF were 83.33% and 94.87%, respectively. Conclusion: GeneXpert MTB/RIF assay can effectively detect MTB and rifampicin resistance in patients with bacteriologically-negative pulmonary TB, demonstrating good diagnostic value for the early identification of bacteriologically-negative pulmonary TB.

Keywords: Bacteriologically-negative pulmonary tuberculosis, mycobacterium tuberculosis, GeneXpert MTB/RIF, rifampicin resistance

Introduction

Tuberculosis (TB) is a highly infectious and life-threatening disease caused by mycobacterium tuberculosis (MTB) [1, 2]. According to the statistics of the WHO, there were 9.6 million cases of TB worldwide with a mortality rate of 15.6% in 2014 [3]. Whether a person can be infected with TB after bacterial invasion depends not only on the number and virulence of the bacteria but also on the immunity of the individual [4, 5]. If the immune system is impaired in the human body, the tubercle bacillus, after entering the body, can multiply quickly to to develop TB [6]. At present, the traditional detection methods for TB bacteria are the acid-fast smear (AFS) and the solid/liquid culture method [7]. However, these methods cannot meet the needs of clinical diagnosis, and their clinical application is highly limited. Bacteriologically-negative pulmonary TB is a type of active TB with negative sputum culture for MTB and negative sputum smear for acid-fast bacillus [8-10]. Patients with bacteriologically-negative pulmonary TB do not show specific clinical characteristics of TB at the early stage of onset. Due to the lack of evidence from bacteriological diagnosis, the diagnosis of bacteriologically-negative pulmonary TB is difficult, and there can be misdiagnosis and mistreatment in clinical practice. Therefore, it is essential to have an effective diagnostic method and a standardized

Figure 1. The diagnosis of patients and the postoperative pathological diagnosis were made by two experienced pathologists. A: Chest CT of a patient with tuberculosis (male, 26 years old). The chest CT revealed tuberculosis with cavity formation in the right upper lobe. The patient received anti-tuberculosis treatment for 9 months and video-assisted thoracoscopic surgery for resection of the right upper lobe. The postoperative pathological examination found necrotizing granuloma in the right upper lobe, considering tuberculosis, the molecular pathological test showed positive results for mycobacterium tuberculosis. B: Chest CT of a patient with lung cancer (female, 28 years old). The chest CT revealed irregular mass shadow with cavity formation in the left upper lobe. Video-assisted thoracoscopic surgery and mediastinal lymphadenectomy were performed. Postoperative pathological examination showed infiltrative adenocarcinoma in the left upper lobe, pT2N0M0. C: Chest CT of a patient with bronchodilation and aspergillus infection.

treatment to prevent the progression of bacteriologically-negative pulmonary TB and to reduce the spread of this disease [11].

GeneXpert MTB/RIF assay is one of the new methods for detecting TB and culture-negative pulmonary TB [12, 13]. This method can also quickly and accurately detect the resistance of mycobacteria to rifampicin in the specimen [14]. However, the samples tested using this method are limited to mainly sputum, cerebrospinal fluid, pleural effusion, pericardial effusion, and other liquid samples, and the application of this method in formalin-fixed paraffin-embedded (FFPE) samples has been rarely reported.

In this study, we aimed to compare the diagnostic efficacy of GeneXpert MTB/RIF assay with those of TB-DNA and AFS tests and to investigate the value of GeneXpert MTB/RIF assay in detecting MTB and rifampicin resistance in FFPE samples.

Materials and methods

Study design and patient enrollment

From October 2015 to October 2018, a total of 125 suspected TB patients who underwent surgery for confirmation of TB or lung cancer at the Department of Thoracic Surgery of Beijing Chest Hospital, Capital Medical University were enrolled in this study. The patients were divided into the TB group (51 males and 32 females, age: 44.06±11.76 years) and the non-TB group (26 males and 16 females, age: 43.61±9.93 years). The TB diagnostic criteria were as follows: 1) the chest CT revealed that patients had the characteristics of TB; 2) patients had typical clinical features of TB; 3) patients complied with the criteria of TB diagnosis defined in the *"Diagnostic and Treatment Guidelines for Tuberculosis"* by the Chinese Society of Tuberculosis, Chinese Medical Association [15]; 4) patients' results of postoperative pathological examination were consistent with the pathological characteristics of TB. In the non-TB group, relevant examination and the postoperative pathological test confirmed no tuberculosis. See **Figure 1**.

The study was approved by Ethics Committee of Beijing Chest Hospital. All patients provided written informed consent.

Methods

GeneXpert MTB/RIF: GeneXpert MTB/RIF assay was performed to detect MTB and rifampicin resistance in FFPE samples. The FFPE samples were placed a centrifuge tube followed by addition of 320 μL of deparaffinization solution, and the samples were incubated at 56°C for 3 min. Next, the samples were treated with 180 μL of ATL buffer and were agitated by a vortex mixer for 10-15 s before high-speed refrigerated centrifugation. Proteinase K (20 μL) was added into the clarified liquid in the tube, and the samples were incubated at 56°C for 1 h and 90°C for 1 h followed by immediate cen-

Table 1. Baseline data of the patients

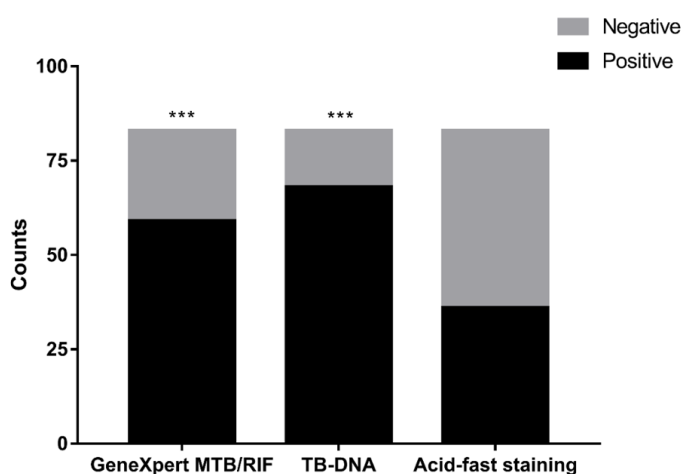| Factor	TB group (n=83)	Non-TB group (n=42)
Gender	51/31	26/16
Age	44.06±11.76	43.61±9.93
BMI	16.82±1.52	18.16±1.93
Confirmed time (months)	20.31±5.15	22.62±6.17
Smoking history	49/34	38/16
Operative methods		
Lobectomy	43	26
Partial lung resection	15	19
Total lung resection	7	5

Note: BMI, body mass index; TB, tuberculosis.

Figure 2. The positive rates of MTB detected by GeneXpert MTB/RIF, TB-DNA, and AFS. ***$P<0.001$ vs. the acid-fast staining. MTB, mycobacterium tuberculosis; TB-DNA, mycobacterium tuberculosis gene amplification test; AFS, acid-fast smear.

trifugation. Finally, 2 mL of pre-reaction solution was added and the samples were centrifuged again. The mixtures were then transferred to a reaction kit for detecting the results of MTB and rifampicin resistance (GeneXpert instrument: Cepheid, USA). MTB results presented as either positive or negative, and the results of rifampicin resistance were interpreted as follows: ΔCT ≥3.5 indicated resistance to rifampicin, and ΔCT <3.5 indicated sensitivity to rifampicin.

MTB gene amplification test (TB-DNA): The nucleic acids were extracted from FFPE samples according to the manufacturer's protocol of the extraction kit, and 200 μL of reagents were added to the PCR tube according to the manufacturer's instructions of the MTB nucleic

acid detection kit (Da'an Gene, China) followed by centrifugation immediately. Real-time quantitative PCR was then performed to detect MTB. If the cycle threshold value ≤39, the specimen was considered to contain tubercle bacilli, and the MTB infection was positive in the tissue; otherwise, the MTB infection was negative in the tissue.

Ziehl-Neelsen (ZN) and Auramine-O (AO) staining and conventional culture of sputum: Each sample was processed by ZO and AO staining for sputum smear microscopy and was conventionally cultured in MGIT Bactec 960 liquid medium (Becton Dickinson, USA). Immune chromatography was applied to isolate the MTB from the liquid culture for rapid identification of the species (Becton Dickinson MGIT™ TBC Identification Test). Phenotypic culture-based drug susceptibility testing method on MGIT 960 (Becton Dickinson, USA) with critical concentrations recommended by WHO was the reference standard for detecting rifampicin resistance [9].

ZN staining for FFPE specimens: ZN staining was performed to detect acid-fast bacillus in FFPE specimens according to the manufacturer's protocol and all AFS slides were reviewed by two pathologists [10].
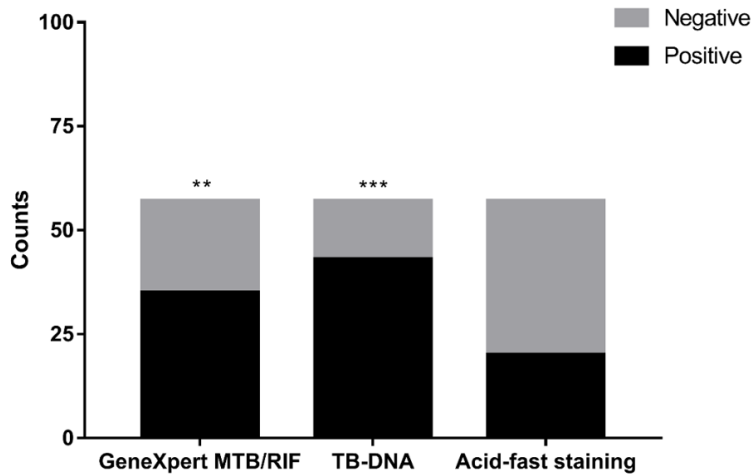
Statistical analysis

SPSS 19.0 statistical software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. The sensitivities, specificities, positive predictive values (PPVs), and negative predictive values (NPVs) of Gene-Xpert MTB/RIF, TB-DNA, and AFS in detecting MTB and rifampicin resistance were analyzed. Measurement data are expressed as mean ± standard deviation. Student's t-test and one-way analysis of variance were used to compare the distributions of continuous variables. Bonferroni test was performed for pairwise comparison. Count data are expressed as number and percentage and compared by Pearson's chi-square test or Fisher's exact test. All statistical analyses were two-sided. $P<0.05$ indicated a statistically significant difference.

Table 2. MTB test results

Method	Result	TB group (n=83)	Non-TB group (n=42)	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)
GeneXpert MTB/RIF	Positive	59	0	71.1 (61.3-80.9)	100	100	63.6 (53.2-74.0)
	Negative	24	42				
TB-DNA	Positive	68	1	81.9 (77.7-86.1)	97.6 (93.0-100)	98.6 (96.1-100)	73.2 (63.7-82.7)
	Negative	15	41				
AFS	Positive	36	1	43.4 (32.7-54.1)	97.6 (93.0-100)	92.3 (86.5-98.0)	53.4 (42.7-64.1)
	Negative	47	41				

Note: TB, tuberculosis; MTB, mycobacterium tuberculosis; TB-DNA, mycobacterium tuberculosis gene amplification test; AFS, acid-fast smear; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

Figure 3. The positive rates of MTB detected by GeneXpert MTB/RIF, TB-DNA, and AFS in the diagnosis of bacteriologically-negative pulmonary TB. **P<0.01 vs. the acid-fast staining, ***P<0.001 vs. the acid-fast staining. MTB, mycobacterium tuberculosis; TB-DNA, mycobacterium tuberculosis gene amplification test; AFS, acid-fast smear.

Results

Baseline data

The baseline data in the TB and the non-TB groups are shown in **Table 1**.

MTB test results

GeneXpert MTB/RIF, TB-DNA, and AFS test methods were used to detect MTB in all the patients, and the positive rates of MTB detected by these methods were 71.08%, 75.90%, 43.37%, respectively. The positive rates of MTB detected by GeneXpert MTB/RIF, and TB-DNA were higher than that by AFS (χ^2=11.912, P<0.001. χ^2=24.740, P<0.001, **Figure 2**). The sensitivities, specificities, PPVs, and NPVs of Gene-Xpert MTB/RIF, TB-DNA, and AFS are listed in **Table 2**.

MTB test results in patients with bacteriologically-negative pulmonary TB

GeneXpert MTB/RIF, TB-DNA, and AFS methods were used to detect MTB in patients with bacteriologically-negative pulmonary TB, and the positive rates of MTB detected by these methods were 61.40%, 75.44%, and 35.09%, respectively. The positive rates of MTB detected by GeneXpert MTB/RIF and TB-DNA were higher than that by AFS (χ^2=6.886 P<0.05, χ^2=17.173 P<0.001); the positive rate of MTB detected by GeneXpert MTB/RIF was lower than that by TB-DNA (P<0.05) as shown in **Figure 3**. The sensitivities, specificities, PPVs, and NPVs of GeneXpert MTB/RIF, TB-DNA, and AFS are listed in **Table 3**.

Resistance to rifampicin in patients with bacteriologically-negative pulmonary TB

From the FFPF tissues of the 57 patients with bacteriologically-negative pulmonary TB, 38 samples from patients with MTB-positive TB were selected. The colonies were taken for drug susceptibility test, and the results showed that 10 patients were resistant to rifampicin, and 28 patients were sensitive to rifampin. Meanwhile, GeneXpert MTB/RIF test showed that 12 patients were resistant to rifampicin, and 26 patients were sensitive to rifampicin. GeneXpert MTB/RIF had a sensitivity of 83.33% and a specificity of 94.87%. See **Table 4**.

Table 3. MTB test results in patients with bacteriologically-negative pulmonary TB

Method	Result	Bacteriologically-negative pulmonary TB (n=57)	Non-TB (n=42)	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)
GeneXpert MTB/RIF	Positive	35	0	61.4% (48.9-73.9)	100%	100%	65.6% (53.2-77.9)
	Negative	22	42				
TB-DNA	Positive	43	1	75.5% (64.3-86.7)	97.6% (93.0-100)	97.7% (93.8-100)	74.5% (63.2-85.8)
	Negative	14	41				
AFS	Positive	20	1	35.1% (22.7-47.5)	97.6% (93.0-100)	95.2% (89.8-100)	52.6% (39.6-65.6)
	Negative	37	41				

Note: TB, tuberculosis; MTB, mycobacterium tuberculosis; TB-DNA, mycobacterium tuberculosis gene amplification test; AFS, acid-fast smear; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

Table 4. Rifampicin resistance (n, %)

GeneXpert MTB/RIF assay	Drug susceptibility test		Total	Sensitivity	Specificity
	Rifampin resistance	Rifampin sensitivity			
Rifampicin resistance	9	3	12	83.33%	94.87%
Rifampin sensitivity	1	25	26		
Total	10	28	38		

Discussion

Bacteriological examination, which is a useful method to find the source of infection, can provide important guidance for the diagnosis of TB and the formulation of chemotherapy [16-18]. However, the sputum examination is not effective for the detection of bacteriologically-negative pulmonary TB, which makes the diagnosis of this disease difficult [19]. It has been estimated that about 70% of the TB cases in China are bacteriologically-negative pulmonary TB [20]. Recently, the molecular biology technique of detecting MTB have been developing rapidly [21]. In addition to the traditional pathological diagnosis, the application of molecular detection based on nucleic acid amplification in FFPE samples can effectively improve the accuracy of the pathological diagnosis of TB [22].

In a study by Chen et al., MTB was detected using GeneXpert MTB/RIF, TB-DNA, AFS, and liquid/solid culture methods, and the sensitivity and specificity of GeneXpert MTB/RIF were 62.43% and 98.42%, respectively [23]. In this study, the sensitivities and specificities of GeneXpert MTB/RIF, TB-DNA, and AFS we found for detecting MTB in patients with bacteriologically-negative pulmonary TB are consistent with the results of the previous research.

The specimens of the patients with culture-negative pulmonary TB showed positive results for MTB detected by GeneXpert MTB/RIF assay, suggesting that GeneXpert MTB/RIF assay is able to detect low MTB content [24]. Since there is high specificity and sensitivity of GeneXpert MTB/RIF assay, this method can be used for the molecular diagnosis of MTB.

Researchers have reported that most of the resistance properties of MTB are caused by resistance-related gene mutations, and the mutation of rpoB is related to rifampicin resistance, with a mutation rate of more than 90% [25, 26]. Since 85%-90% of rifampicin-resistant MTBs is also resistant to isoniazid, and mutation detection of rpoB resistance genes is of great significance for the screening of multi-drug-resistant tuberculosis [27]. GeneXpert MTB/RIF assay is a semi-nested real-time quantitative PCR method that targets rpoB to detect rifampicin resistance. In a study by Jahan et al., GeneXpert MTB/RIF assay was applied to evaluate the accuracy in detecting rifampicin resistance, and its sensitivity and specificity were 98.52% and 100%, respectively [28]. In our study, the sensitivity and specificity of GeneXpert MTB/RIF assay for detection of rifampicin resistance were 83.33% and 94.87%, respectively, which is consistent with the previous research results. Our results suggested that GeneXpert MTB/RIF can be a suitable method for detecting rifampicin resistance in patients with bacteriologically-negative pulmonary TB.

There were still some limitations in our study. The study was a single-center study with a small sample size which may lead to biased results. Therefore, a multicenter study with a larger sample size needs to be performed in the future. Also, a joint analysis of the three testing methods needs to be carried out.

In summary, the effective detection of MTB and rifampicin resistance by GeneXpert MTB/RIF assay in FFPE specimens may bring some new insights into the diagnosis of TB, especially bacteriologically-negative TB and rifampicin-resistant TB.

Disclosure of conflict of interest

None.

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