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Short Communication

Comparison of Real-time PCR Fluorescence Melting Curve Analysis and Xpert MTB/RIF for Detection of Rifampicin Resistant Mycobacterium Tuberculosis from Patients with Extra-pulmonary Tuberculosis

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Abstract

Objective: Compare the real-time fluorescent Polymerase Chain Reaction (PCR) melting curve analysis (MCA) with Xpert MTB/RIF for detection of rifampicin resistant Mycobacterium tuberculosis (RRTB) from extra-pulmonary tuberculosis (EPTB).

Methods: The medical records of patients with EPTB were reviewed and collected from February 2017 to February 2019. The samples were tested by drug susceptibility tests. Taking the rifampicin resistance (RR) result of proportional method as the gold standard, the sensitivity, specificity and coincidence rate of MCA and Xpert method to detect RR was calculated.

Results: There were 134 samples enrolled in this study including 75 males and 59 females. The results of RR were consistent with MCA and Xpert methods in 123 cases. 106 cases were consistent with results of the proportional method detection. Taking the proportional method as the standard, the sensitivity, specificity, positive predictive value, negative predictive value and coincidence rate of Xpert method to detect RR were 95.2%, 83.7%, 72.7%, 97.5%, 87.3%; the MCA method were 90.5%, 73.9%, 61.3%, 94.4% and 79.1% respectively. There were significant differences on the detection of RIF sensitivity in the different types of patients detected by two methods (P<0.01).

Conclusion: Xpert and MCA had high sensitivity and specificity in detecting surgical specimens of patients with EPTB and were suitable for early and rapid detection of RRTB from EPTB.

Keywords: Mycobacterium tuberculosis, extra-pulmonary, Real-time PCR, Fluorescence MCA, Xpert MTB/RIF, RR

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1 INTRODUCTION

Tuberculosis is an important global public health threat to human health, especially multiple-drug-resistant tuberculosis (MDR-TB). According to the World Health Organization, in the worldwide there were an estimated 10.60 million new cases and approximately 1.30 million deaths in 2022. Among them there were 560,000 new-onset patients with rifampicin resistant Mycobacterium tuberculosis (RRTB) and 460,000 MDR-TB patients. In China about 73,000 patients were RRTB that account for 13% of the world[1]. MTB drug resistance is the main cause of death of tuberculosis patients, and then the timely diagnosis of drug-resistant tuberculosis is very important for the prevention and control of tuberculosis[2].

The traditional susceptibility test is based on culture. It takes about 4 weeks to obtain the drug susceptibility test results after 3-8 weeks of cultivation and isolation of strains. It takes a long time and reliable drug sensitivity test result cannot be provided quickly. The real-time Polymerase Chain Reaction (PCR) with fluorescence melting curve analysis (MCA) and Xpert method are used to quickly detect RRTB. MCA and Xpert are molecular biological detection methods used in our country's tuberculosis laboratory in recent years, and the main specimen is strain or sputum[3-6]. In order to clarify the clinical value and significance of these two methods for detection of extra-pulmonary tuberculosis (EPTB) samples in Chongqing, this study compared the sensitivity and specificity of the test results of two methods for the detection of EPTB samples.

2 METHODS

2.1 Patients

From February 2017 to February 2019 the medical records were reviewed who were hospitalized in hospital. The patients with EPTB who had drug susceptibility tests (DST), MCA and Xpert results for detection of rifampicin resistance (RR) were included in this study.

2.2 Ethical Statement

Informed consent was obtained and had been written from all patients who agreed that the necrotic tissue or pus would be tested and the detection results would be used for research before surgery and treatment. This research was a retrospective study and approved by the IRB of Chongqing Public Health Medical Center and Tianjin First Central Hospital.

2.3 Specimen Collection and Pretreatment

Cerebrospinal fluid, pleural and abdominal fluid, pus or puncture fluid and tissue removed from the lesion during the operation were collected by the clinician, preserved at low temperature (4-8℃), and sent for examination in time. Urine, pleural fluid, and ascites should not be less than 20mL, and cerebrospinal fluid should not be less than 2mL. The specimens were decontaminated using the N-acetyl-l-cysteine-sodium hydroxide (NALC-NaOH) method[7]. The processed sediment was washed using a sterile 0.9% NaCl solution, re-suspended in 1.5 mL sterile 0.9% NaCl solution and then equally divided into three thirds.

2.4 Specimen Separation and Culture

One of the three thirds was centrifuged and the sediment was inoculated in both Bactec MGIT 960 system (Bacton Dickinson and Company, MD) and neutral Roche medium (Zhuhai Encode Medical Engineering Co., Ltd). The culture was regarded as positive if one or both of the above two culture methods produced positive results.

2.5 Strain Identification

Colloidal gold method was used to detect the MPB 64 antigen of mycobacterium tuberculosis filtrate protein in mycobacterium culture products. 100μl treated culture samples were added and the results were observed after 15min. If purplish red bands appeared on both the detection line and the quality control line, the strain was identified as mycobacterium tuberculosis compound group (positive). If there are no purplish red bands on the detection line but purple red bands on the quality control line, the strain will be judged as non-tuberculous Mycobacterium (negative).

2.6 DST

The strain identified as MTB by the colloidal gold method was carried out the susceptibility test of rifampicin by the indirect proportional method. The final concentration of rifampicin in this method was 40μg/mL. The judgment standard of the drug resistance result: the drug resistance rate>1%, and the tested strain was considered to be resistant to the drug.

2.7 MCA Detection

This method detects directly mutations of the 27 amino acid codon regions in MTB rpoB gene 507-533. Grind the specimen, add 1 to 2 volumes of 4% NaOH for digestion, mix evenly, leaves at room temperature for 60min. Take 2mL of the mixed solution and centrifuge on a low-temperature high-speed centrifuge with a centrifugal radius of 8.8cm and 13,000rpm, 5min, discard the supernatant, add 1mL of the sputum treatment solution from matching the kit to the precipitate, and transfer to a metal bath at 100℃ for 10min. Heat-sterilized samples were subjected to nucleic acid extraction, and the purified DNA was collected and stored at -20℃. The DNA samples were left to thaw at room temperature and centrifuged at a low temperature and high speed centrifugal radius of 8.8cm at 13,000rpm for 2min. Take the supernatant for PCR test. The test result is automatically read by the supporting software and is divided into "no MTB detected", "unclear", "sensitive", and "resistant". If the drug resistance result is "uncertain", it will not be

Table 1. Demography of 134 Patients with EPTB

Characteristics	N	Proportion (%)	*P*
Mean age (y)	32.8±14.1	-	
Male/Female	75/59	56.0/44.0	
Samples			
Lymph node (M/F)	21/22	15.7/16.4	
Cold abscess (M/F)	22/13	16.4/9.7	
Surgically removed tissue (M/F)	20/10	14.9/7.5	
Pleural fluid (M/F)	6/3	4.5/2.2	
Wound secretion (M/F)	2/5	1.5/3.7	
Cerebrospinal fluid (M/F)	1/3	0.7/2.2	
Urine (M/F)	0/3	0/2.2	
Stool (M/F)	2/0	1.5/0	
Peritoneal fluid (M/F)	1/0	0.7/0	
Patients			
New cases (M/F)	43/32	32.1/23.9	>0.05
Retreatment (M/F)	32/27	23.9/20.1	

included in this study. In the process the purified DNA was divided into three equal parts, the first was for MCA detection, the second was for detection again when the results of MCA and Xpert were inconsistent and the third was for sequencing if the two results of MCA detection were inconsistent.

2.8 Xpert Detection

Different types of samples have different pretreatment methods: surgical tissue or pus specimens were ground into a uniform paste for use; urine, pleural and abdominal fluid and cerebrospinal fluid were centrifuged in a 3,000g low-temperature centrifuge for 20-30min, then supernatant were discarded and precipitation left; feces and saturated normal saline should be mixed, and suspension were kept for 2-5min by full oscillation, the topmost suspended solids were mixed with 3-5 times volume of PBS, centrifuged at 3,000g for 20-30min, and the supernatant was discarded for later use. After the above pretreatment, add the sample treatment solution matching the kit with 1-2 times the volume for further digestion and treatment, mix evenly, and store at room temperature for no less than 60min before testing.

Xpert uses 6 molecular beacons to simultaneously detect 6 kinds of probes, of which 5 overlapping molecular beacon probes (A, B, C, D, E) selectively cover the 81bp core region of rpoB gene of rifampicin resistant determining region (RRDR), 1 probe as an internal control to detect whether it has a mutation, and determine whether it is infected with MTB and resistant to rifampicin. Grind the specimen, add 1 to 2 times the volume of the sample processing solution matching the kit for digestion, mix evenly, and place it at room temperature for not less than 60min before it can be tested on the machine. The Xpert test results are automatically interpreted by the supporting software system, and the results are divided into undetected (negative), extremely low (positive), low (Positive), moderate (positive), high (positive). RR test results are divided into "drug resistance", "sensitive" and "uncertain". The result of the "unclear" specimen was not included in this study.

2.9 Sequencing and Comparison of DNA Samples

DNA of negative culture samples without DST results and inconsistent results of MCA and Xpert detection were sequenced.

2.10 Statistical Analysis

SPSS 22.0 analysis software was used for statistical analysis. The sensitivity, specificity, positive predictive value and negative predictive value of the MCA and Xpert methods were compared with the DST with indirect proportional method. $P<0.05$ was considered statistically significant.

3 RESULTS

There were 134 samples were detected by proportional method, MCA and Xpert. Fifty-four samples were detected by MCA and Xpert due to negative culture results. The demography of 134 patients was displayed in the Table 1. There was no significant difference in the gender distribution between the new cases and retreatment.

3.1 Detection Results of MCA and Xpert

Of the 134 samples there were consistent results in the 106 samples detected by the proportional method, MCA and Xpert, which included 38 cases of RR and 68 cases of rifampicin sensitivity; the rest 28 samples were inconsistent detection results by the above three methods. Taking the proportional method as the standard, the sensitivity, specificity, positive predictive value and negative predictive value of Xpert and MCA to detect RR are showed in the Table 2.

3.2 Detection Results of MCA and Xpert in the Different Types of Patients

There were significant differences on the detection of RIF resistance in the different types of patients detected by two methods ($P<0.01$) (Table 3). The sensitivity and resistance were 85.3% and 14.7% in the new cases using the MCA, and 86.7% and 13.3% using the Xpert. The sensitivity and resistance were 15.7% and 84.3% in the retreatment cases using the MCA, and 23.7% and 76.3% using the Xpert.

3.3 Analysis of Inconsistent Results

The MCA and the proportion method for detecting RR results were inconsistent in 28 patients, of which 24 were sensitive to the proportion method, while the MCA showed rpoB amino acid codon mutations from 529 to 533 and/or rpoB mutations at positions 521 to 528; the results of the proportion method in 4 cases were drug resistance but no mutation was detected by the MCA. There were 17 inconsistent results of rifampin resistance detection by

Table 2. Comparison of the Results of Rifampin Resistance Detected by the Two Methods with Drug Sensitivity Test by the Proportional Method as the Standard (*n*=134)

	No. of cases with Proportional method		*P*	Mean (%)			
	Resistance	Sensitivity		Sensitivity	Specificity	PPV	NPV
MCA							
Resistance	38	24	<0.05	90.5%	73.9%	61.3%	94.4%
Sensitivity	4	68					
Xpert							
Resistance	40	15	<0.05	95.2%	83.7%	72.7%	97.5%
Sensitivity	2	77					

Table 3. Comparison of Rifampin Resistance Detected by the Two Methods in Different Type Cases (*n*=134)

	No. of the different type cases		*P*
	New cases (*n*=75)	Retreatment (*n*=59)	
MCA			
Resistance	11	51	<0.01
Sensitivity	64	8	
Xpert			
Resistance	10	45	<0.01
Sensitivity	65	14	

Xpert and proportion method, of which 15 were sensitive by proportion method, while Xpert showed drug resistance because some molecular signal probes were not detected; 2 proportion method results were drug resistance but the Xpert detected all molecular signal probes and showed sensitivity.

3.4 DNA Sequencing

Fifty-four specimens were unable to obtain the DST results detected by indirect proportion method due to the negative culture results and detected by MCA and Xpert. Of 54 specimens 31 cases were RR and were consistent results detected by Xpert and MCA. The other 23 cases were inconsistent results detected by Xpert and MCA and were displayed in the Table 4.

4 DISCUSSION

The result of rifampicin susceptibility test is one of the important indicators to assess whether MTB is MDR-TB. The phenotypic DST of MTB is the gold standard susceptibility method. The main methods include MGIT 960, solid absolute concentration method and proportional method. The solid absolute concentration and proportional methods require a longer time, and the detection time of MGIT 960 method has been greatly shortened but the cost of instruments and reagents is relatively high, which limits its range of use[8,9].

Xpert is one of the methods for rapid detection of MTB and rifampin resistance in recent years. This detection technology based on the principle of semi nested real-time fluorescent quantitative PCR, designed primers and probes according to the rpoB gene 81bp RRDR to test the mutated, can directly detect MTB and the rifampicin resistant (rpoB sequence mutation). There were some studies that showed the sensitivity and specificity of this method was higher than existing detection methods in detecting sputum and some other types of specimens[5,6]. The Xpert integrates the three steps of sample preparation, amplification and detection required by traditional PCR detection, automatically provides the samples to be tested into GeneXpert MTB/RIF reaction box, and the system will automatically perform nucleic acid extraction, amplification and target sequence detection, which is easy to operate and suitable for clinical application.

The MCA was used to detect the mutation of rpoB gene 81bp in the RR determining region of Mycobacterium tuberculosis complex group for resistance screening. The sequence mutation information was obtained by acquiring melting point (Tm value) that is from the fusion curve of hybrid product of the probe and the sequence. The whole process takes 3-4 hours and save time than the traditional method by culture positive strain and drug sensitivity test, and nucleic acid extraction is high degree automation, save manpower than the traditional method. In China the MCA method has been used for detection of the RR and the sensitivity was 94.2%[10], but there were few reports for the detection of EPTB.

Xpert and MCA method had high detection sensitivity and specificity of sputum sample from patients with PTB, and the sensitivity and specificity of Xper method were higher than those of melting curve method. But according to studies the sensitivity and specificity of the above methods were slightly lower than those of proportional method[2-4]. The inconsistent detection results of MCA or Xpert and proportion method may be due to the minimum limits of detection concentration of wild type or mutant type. Genotype detection only detects MTB drug resistance decided by rpoB gene RRDR. RR caused by other genes or gene region mutation and other RR mechanism cannot be detected by percentage method. A strain detected by Xpert was sensitive to rifampicin, which may be RR detected by proportional method because of heterogeneous drug resistance or fast-growing MTB. Some studies showed the sensitivity of GeneXpert MTB/RIF detection technique in the diagnosis of EPTB was 25.0%~96.6% and the specificity was close to 100%, which was similar to our results[3-6]. That indicated that this technique has high clinical value in the diagnosis of EPTB. However, GeneXpert MTB/RIF has a high heterogeneity in the sensitivity and specificity of

Table 4. DNA Sequencing of Samples of Negative Culture and Inconsistent of MCA and Xpert Detection (*n*=23)

MCA	Xpert		DNA Sequencing Results
	Resistance	Sensitivity	
Resistance			
		9	Wild type
		2	amino acid mutations: 513 CAA→CTA, 532 GCG→GCA
		2	base sequence mutation but no amino acid change
Sensitivity			
		8	Wild type
	2		Wild type

samples from thoracic and abdominal water, cerebrospinal fluid, pericardial effusion, which requires more extensive and in-depth studies to reveal the causes. According to the research on GeneXpert MTB/RIF in children the sensitivity and specificity were 54.0% and 93.8% on the detection of tuberculous meningitis, 90.4% and 89.8% on the lymph node tuberculosis, 90.0% and 98.3% on the RR[11]. According to report, the Pooled Xpert sensitivity (defined by culture) varied across different types of specimens (31% in pleural tissue to 97% in bone or joint fluid); Xpert sensitivity was >80% in urine and bone or joint fluid and tissue. Pooled Xpert specificity (defined by culture) varied less than sensitivity (82% in bone or joint tissue to 99% in pleural fluid and urine). Xpert specificity was ≥98% in cerebrospinal fluid, pleural fluid, urine, and peritoneal fluid. On the Xpert testing for RR, Xpert pooled sensitivity (20 studies, 148 specimens) and specificity (39 studies, 1,088 specimens) were 95.0% (89.7% to 97.9%) and 98.7% (97.8% to 99.4%), respectively. For a population of 1000 people where 120 have rifampicin-resistant TB, 125 would be positive for rifampicin-resistant TB: of these, 11 (9%) would not have RR (false-positives); and 875 would be negative for rifampicin-resistant TB: of these, 6 (1%) would have RR (false-negatives)[12]. Although there were some researches on sensitivity and specificity of Xpert for detection of EPTB and RR, no report of Xpert on the different types of patients. In our study MCA and Xpert were the same detection capability in the new and retreatment cases.

23 cases with a negative culture results, without detection results of RR by proportion method and inconsistent detection results of MCA and Xpert were DNA sequenced. Of 21 cases with RR by MCA detection and rifampicin sensitive by Xpert, re-inspection results of 8 cases were "sensitive" and were inconsistent with the results for the first time. The first results was "resistance" due to the MCA was in bimodal or fused peaks, software automatic identification system judged hybrid samples, was "resistance", but in the end no mutation was confirmed by sequencing. The retest results of 9 cases were "drug resistance" by the MCA, which was consistent with the first result. The sequencing confirmed that there was no mutation site. The MCA showing bimodal or fused peaks may be due to the drug resistance caused by low heterogeneity, the sequencing indicated "wild type". This phenomenon suggested that the composition of surgical samples from EPTB was complex and most of the samples were mixed with blood. In the detection of MCA, it was easy for the bimodal or fused peaks to be misjudged as "drug resistance". It is suggested that the detection of hybrid samples should be repeated so as to accurately determine the drug resistance of the samples. 2 cases re-inspection result by MCA were "resistance", consistent with the results for the first time, confirmed by sequencing base sequence change but not cause amino acid mutation, which may be that the MCA screened nucleic acid sequence, not the amino acid sequence. No amino acid changing mutations may be convicted of drug resistance, causing false positive. According to research the mutation of Leu511Pro, Leu533Pro, Asp516Tyr and His526Asn in the rpoB region were the main reasons for the inconsistency between the genotypic and phenotypic results for MTB susceptibility to rifampicin[13]. Restricted by many factors, such as phenotypic DST, molecular DST and other methods, the detection principle is different, some gene mutations do not affect phenotypic drug resistance, and the gene mutations of drug resistance determining region can not completely regard phenotypic DST as drug resistance. Phenotypic DST of resistance and sensitive strains mixture was resistance, but when mixture contained low proportion of drug-resistant strain, may cause the melting peak of MCA consistent with positive controls, and the test strain was judged to be sensitive, appeared false negative.

MCA method is invented and applied for a patent by Chinese researcher. At same time MCA was applied to detect the mutation scanning, mutation identification and mutation genotyping[14]. This method has been validated by other researchers as effective in detecting DRTB mutations[15], but there were few reports about the DR-EPTB mutations using MCA method. This study demonstrated the MCA methods was easy to design, cheap to synthesize, amenable to color multiplexing, and compatible to different platforms for detection of DR-TB mutations and confer cross-platform compatibility on major real-time PCR instruments. This method should be widely used and promoted by the laboratory in China.

5 CONCLUSION

Xpert and MCA had high sensitivity and specificity in detecting surgical specimens of patients with EPTB, and were suitable for early and rapid detection of RRTB from EPTB.

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Conflicts of Interest

The authors declared no conflict of interest.

Authors Contribution

Li T and Shi T were responsible for the conception and design. Wang P, Li Q, Zhang H, Jiang L and Yang L were responsible for Collecting the data. Li T and Shi T were responsible for analyzing the data. Ying H wrote the paper. Li T and Shi T interpreted the results. Li T and Ying H were responsible for obtaining funding. All authors have read and approved the final manuscript.

Abbreviation List

DST, Drug susceptibility test
EPTB, Extra-pulmonary tuberculosis
MCA, Melting curve analysis
MDR-TB, Multiple-drug-resistant tuberculosis
PCR, Polymerase Chain Reaction
RR, Rifampicin resistance
RRDR, Rifampicin resistant determining region
RRTB, Rifampicin resistant Mycobacterium tuberculosis

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