Original article

Fluorescent quantitative PCR detection of *Mycobacterium tuberculosis* in tissue sections from granulomatous lesions retrieved using EDTA

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ABSTRACT

Aims This study aimed to use EDTA to retrieve paraffin-embedded tissue sections of inflammatory granulomatous lesions and increase the detection rate of tuberculosis (TB)/non-tuberculous mycobacteria. Due to the influence of chemical reagents during the fixation process, the amplification of fluorescent quantitative PCR was blocked after DNA extraction, and the results were not ideal.

Methods Special staining technologies (acid-fast and Auramine O) and fluorescent quantitative PCR were used to detect TB/non-tuberculous mycobacteria in 125 cases of inflammatory granulomatous lesions in paraffin-embedded tissue sections with and without EDTA retrieval.

Results In 125 cases of inflammatory granulomatous lesions, 75 cases (60%) were positive for mycobacteria using fluorescent quantitative PCR without EDTA retrieval, of which 74 cases (59.2%) were detected with TB mycobacteria and 1 case (0.8%) with non-tuberculous mycobacteria. The average cycle threshold value of positive specimens ranged from 29 to 32 (30.5). However, 88 cases (70.4%) were positive for mycobacteria using fluorescent quantitative PCR with EDTA retrieval, of which 83 cases (66.4%) were detected with TB mycobacteria and 5 cases (4%) with non-tuberculous mycobacteria. The average Ct value of positive specimens ranged from 27 to 30 (28.0). Statistical differences were found between the two groups ($p < 0.05$; $p < 0.01$).

Conclusions This study showed that compared with special staining technologies (acid-fast and Auramine O) and molecular pathology detection, fluorescent quantitative PCR with EDTA retrieval could greatly improve the detection rate of TB/non-tuberculous mycobacteria and increase the sensitivity of the fluorescent quantitative PCR.

INTRODUCTION

Currently, tuberculosis (TB) is one of the most infectious diseases, and according to the estimates of the WHO,[1] almost a third of the world population is infected with *Mycobacterium tuberculosis*. During 2006, the number of new TB cases in the world was up to 9.2 million, which included 4.1 million highly infectious cases whose sputum smears were positive and 1.7 million death cases. It means that about 4600 people died from TB every day. In 2010, the fifth epidemiological sampling for TB showed that about 1.3 million TB cases were detected per year, which was about 14.3% of the global estimate and second in the world. The prevalence of active TB was 459/0.1 million, of which the prevalence of TB with positive sputum smears was 66/0.1 million and the prevalence of bacteria-positive TB was 119/0.1 million. So, the epidemic situation was still very serious.[2]

The pathological diagnosis was thought to be the standard for TB diagnosis, but atypical tuberculous lesions appeared frequently in the daily workup, which needed to be differentiated from other lesions such as sarcoidosis, Crohn's disease and fungal granuloma. The use of acid-fast and Auramine O staining or fluorescent quantitative PCR to detect *M. tuberculosis* could provide powerful evidence in the pathological diagnosis of atypical tuberculous lesions.

The use of acid-fast and Auramine O staining showed the detection rate of tuberculous mycobacteria was lower. The detection rate of TB was 31%–50.1% and 40.5%–65.7% using Ziehl-Neelsen acid-fast staining and Auramine O staining, respectively.[1–5] The detection rate of tuberculous mycobacteria using fluorescent quantitative PCR in frozen tissue sections of TB lesions was up to 75.8%.[6–8] However, the tuberculous mycobacteria detection rate was just 50%–60% when the same method was used in the tissue sections of granulomatous lesions that were formalin fixed and paraffin embedded.[3] Sometimes tuberculous mycobacteria could not be detected in paraffin-embedded specimens of typical TB in our work.

Some researchers thought that the DNA of tissues or bacteria in paraffin-embedded sections would be damaged to a certain degree. Targeted DNA of tissues could be ruptured and missed during the fixation process, which might lead to false-negative results.[9] Other researchers believed that it might be related to tissue protein cross-linking during formalin fixation.[10 11] High-temperature and high-pressure retrieval and EDTA heat-mediated retrieval were the representative retrieval schemes for antigen retrieval in immunochemistry (IHC), the principle of which was that the protein cross-linking induced by formaldehyde was broken by heating. So, the antigen sites were exposed and contributed to the success of IHC.[6 7 12 13]

The detection rate of TB using fluorescent quantitative PCR in paraffin-embedded sections was reported to be 50%–75.8%.[1–3] Although the specificity of this method was good and the detection rate was higher, some results still were false negative.[1 2] Thus, ways to improve the detection rate were urgently needed. Some scholars stated that formalin fixation causes protein cross-linking and reduces the detection rate of *M. tuberculosis* in

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tissue sections.[4 5] The antigen retrieval could open the tissue protein cross-linking caused by formalin fixation and significantly increase the detection rate of IHC.[6] To verify that the EDTA heat-induced retrieval could improve the mycobacterial detection rate using fluorescence quantitative PCR, *M. tuberculosis* was detected in this study using fluorescent quantitative PCR in paraffin-embedded tissue sections of granuloma lesions retrieved with EDTA, and compared with the results of routine fluorescence quantitative PCR.

This study attempted to introduce antigen retrieval into the process of fluorescent quantitative PCR and acid-fast and Auramine O staining, and hoped to increase the detection rate of TB/non-tuberculous mycobacteria. The samples were put into an Eppendorf (EP) tube, dewaxed and retrieved with EDTA; then, DNA was extracted, and fluorescence quantitative PCR detection was carried out. Also, acid-fast staining and Auramine O staining were performed after heat-induced retrieval of the samples with EDTA. The results showed that heat-induced retrieval with EDTA could greatly increase the detection rate of TB/non-tuberculous mycobacteria.

METHODS
Samples
A total of 125 specimens of tuberculous granuloma with H&E staining were collected from February 2014 to May 2014 in the Department of Pathology of the Fuzhou General Hospital of Nanjing Military Command. This study was approved by Fuzhou General Hospital ethics committee. It included 83 males and 42 females with the mean age of 45.3 years (range 26–65 years). The specimens were taken by lung puncture or bronchial fiberscope biopsy (58 cases), lymph node biopsy (52 cases), lung resection (10 cases) and skin biopsy (5 cases). Lymph node tissues of non-granulomatous lesions (30 cases) were used as the negative control. Some patients were selected for treatment and follow-up.

Methods
The slicing of all specimens was done in an airtight biological safety cabinet, and a separate microtome knife was used for each specimen. All specimens were soaked in 95% ethanol solution for 5 min and then dried. A total of 20 sections, 6 μm thick, were prepared from each, 10 of which were used for routine fluorescence quantitative PCR and another 10 for fluorescence quantitative PCR with EDTA heat-induced retrieval to detect TB/non-tuberculous mycobacteria. Another two serial sections were used for acid-fast staining and Auramine O staining, respectively, following the method described in previous studies.[3 7]

Instruments and reagents
Automatic real-time fluorescent quantitative PCR detector was procured from Applied Biosystems (Carlsbad, California, USA). DNA extraction reagent for biopsy tissue was obtained from the MagCore (Taipei, China). A mycobacterial nucleic acid detection kit for fluorescence quantitative PCR was purchased from the CapitalBio in Beijing, China (No. 301031). The antigen retrieval liquid was 0.001 mol/L sodium EDTA (pH 9.9).

Fluorescence quantitative PCR for TB/non-tuberculous mycobacteria detection
DNA extraction for routine fluorescence quantitative PCR detection
Ten 6 μm sections were put into an EP tube and 1 mL of xylene was added for dewaxing. After mixing for 10 s and centrifuging

for 5 min at room temperature, the supernatant was discarded. The remnant was vortexed after adding 1 mL of anhydrous ethanol. The supernatant was again discarded after centrifuging for 5 min at room temperature. The precipitate was dried at room temperature or 37°C. Then, 400 μL of buffer and 20 μL of proteinase K were added to the precipitate. The mixture was incubated for 100 min at 56°C and 30 min at 90°C, cooled to ambient temperature and then transferred to the magnetic bead extraction apparatus for extracting DNA after filtering.

Florescence quantitative PCR detection after EDTA heat-induced retrieval
Ten 6 μm sections were put into an EP tube, and 1 mL of xylene was added for dewaxing. After mixing for 10 s and centrifuging for 5 min at room temperature, the supernatant was discarded. The remnant was vortexed after adding 1 mL of anhydrous ethanol. The supernatant was again discarded after centrifuging for 5 min at room temperature. The precipitate was dried at room temperature or 37°C. Then, 1 mL of EDTA retrieval liquid was added to the precipitate. The mixture was put in a metal bath (95°C) for 10 min. The supernatant was discarded after centrifuging the mixture for 5 min at room temperature; 1 mL of distilled water was added for washing. The supernatant was again discarded after centrifuging for 5 min. Then, 400 μL of buffer and 20 μL of proteinase K were added to the precipitate. The mixture was incubated for 100 min at 56°C and 30 min at 90°C, cooled to ambient temperature and

Table 1 Comparison between mycobacterial detection results of fluorescent quantitative PCR and acid-fast and Auramine O staining in 125 cases of inflammatory granuloma

Method	Positive number for acid-fast staining (%)*	Positive number for Auramine O staining (%)†
Fluorescent quantitative PCR detection—positive	37/75 (49)	46/75 (61)
Fluorescent quantitative PCR detection—negative	9/50 (18)	15/50 (30)
Fluorescent quantitative PCR detection after EDTA retrieval—positive	46/88 (52)	61/88 (69)
Fluorescent quantitative PCR detection after EDTA retrieval—negative	0/37 (0)	0/37 (0)

*Comparison among groups, p<0.05.
†Comparison among groups, p<0.01.

Table 2 Comparison between mycobacterial detection results of two fluorescent quantitative PCR methods in 125 cases of inflammatory granuloma

Method	Positive case number*	Per cent*	Average value of Ct gene amplification of positive cases
Fluorescent quantitative PCR detection	75	60.0	29–32 (30.5)
Fluorescent quantitative PCR detection after EDTA retrieval	88	70.4	27–30 (28.0)

*Comparison among groups, p<0.05.
†Comparison among groups, p<0.01.

then transferred to the magnetic bead extraction apparatus for extracting DNA after filtering. https://dict.youdao.com/w/metal_bath_treatment/—#

Fluorescent quantitative PCR detection

Three groups (negative control, positive control and blank control) were set. Template DNA (2 µL) was added into a reaction tube and incubated for 300 s at 37°C. PCR was performed with initial denaturation at 94°C for 3 min, followed by 45 cycles of amplification (at 94°C for 15 s and 60°C for 30 s), and final extension at 50°C for 10 s. Carboxyfluorescein (FAM) and green fluorescent protein (VIC) detection channels were chosen at the same time. The FAM channel detected TB mycobacteria, and the VIC channel detected 16 kinds of non-tuberculous mycobacteria (*M. intracellulare*, *M. avium*, *M. gordonae*, *M. kansasii*, *M. fortuitum*, *M. phlei*, achromatic mycobacterium, *M. scrofulaceum*, *M. gilvum*, *M. terrae*, *M. chelonae/abscessus*, *M. marinum/ulcerans*, *M. szulgai/malmoense*, *M. xenopi*, *M. aurum* and *M. smegmatis*). Fluorescence collection point was at 60°C for 30 s, and the results were analysed according to the reagent instruction. If Ct value was >40, the result was considered as negative.

Statistical analyses

The data were analysed using the SPSS V.19.0 statistical software (SPSS, Chicago, Illinois, USA). The comparison between the two groups was assessed using χ^2 test. A p value <0.05 was considered as statistically significant.

RESULTS

Of 125 specimens, 46 cases (36.8%) and 61 cases (48.8%) of acid-fast bacilli were detected using acid-fast staining and Auramine O staining, respectively (table 1). A total of 75 specimens (60%) were positive for mycobacteria using fluorescent quantitative PCR detection (table 2), of which 74 cases (59.2%) were detected with TB mycobacteria and 1 case (0.8%) with non-tuberculous mycobacteria. The average Ct value of positive specimens ranged from 29 to 32 (30.5) (figure 1).

Of 125 specimens, 88 specimens (70.4%) were positive for mycobacteria using fluorescent quantitative PCR with EDTA retrieval (table 2), of which 83 cases (66.4%) were detected with TB mycobacteria and 5 cases (4%) with non-tuberculous mycobacteria. The average Ct value of positive specimens ranged from 27 to 30 (28.0) (figure 1). The differences between the two groups were statistically significant (table 2, *p<0.05, †p<0.01). The results of 30 controls were all negative.

DISCUSSION

The pathological diagnosis was thought to be standard for TB diagnosis, but atypical tuberculous lesions appeared frequently in daily workup, which needed to be distinguished from other lesions (such as sarcoidosis, Crohn's disease and fungal granuloma).
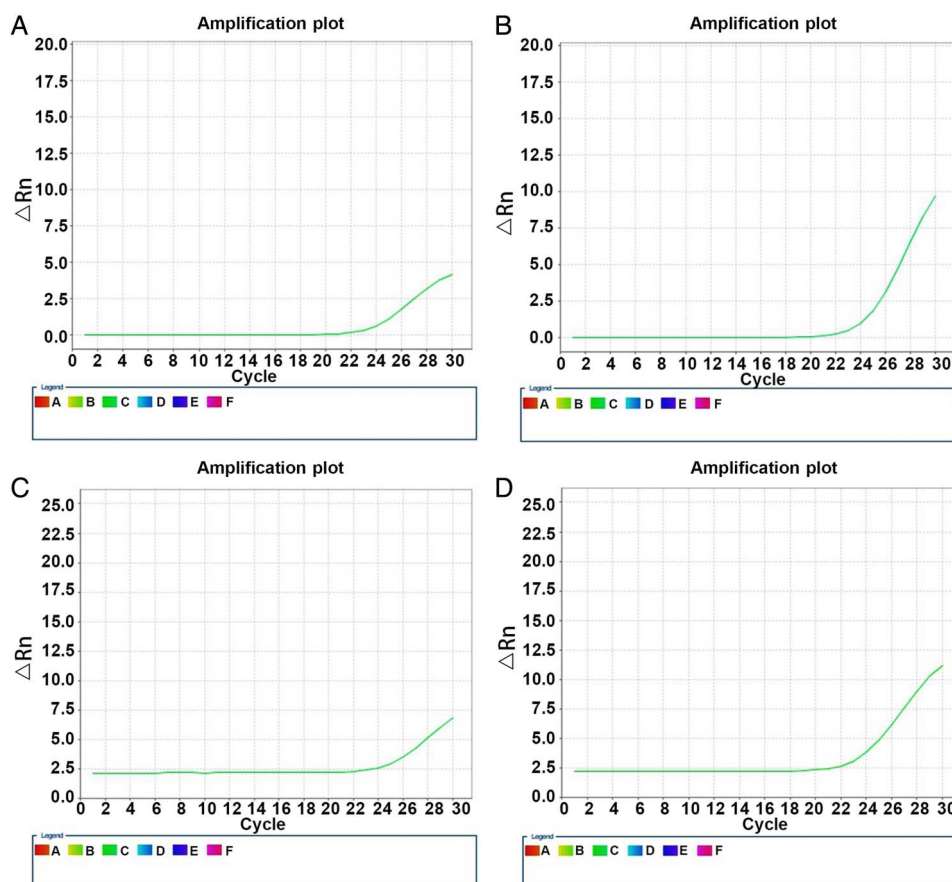
Figure 1 Amplification plots of mycobacteria in granulomas using two fluorescent quantitative PCR detection methods. (A) Amplification plot of tuberculosis (TB) mycobacteria in routine fluorescent quantitative PCR detection. (B) Amplification plot of TB mycobacteria in fluorescent quantitative PCR detection after EDTA retrieval. (C) Amplification plot of non-tuberculous mycobacteria in routine fluorescent quantitative PCR detection. (D) Amplification plot of non-tuberculous mycobacteria in fluorescent quantitative PCR detection after EDTA retrieval.

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Table 3 Mycobacteria detection results of fluorescent quantitative PCR detection before/after EDTA retrieval in 125 cases of inflammatory granuloma with different specimen types

Specimen type	Positive number of fluorescent quantitative PCR detection (%)*	Positive number of fluorescent quantitative PCR detection after EDTA retrieval (%)†
Lung puncture or bronchial fiberscope biopsy	37/58 (64)	43/58 (74)
Lymph node biopsy	35/52 (67)	38/52 (73)
Lung resection	2/10 (20)	5/10 (50)
Skin biopsy specimens	1/5 (20)	2/5 (40)
Lymph node tissues of non-granulomatous lesions	0/30 (0)	0/30 (0)

*Comparison among groups, p<0.05.
†Comparison among groups, p<0.01.

As histological sections may not completely show typical histological characteristics, it was difficult to identify tuberculous granulomas. Thus, besides the histological identification, the methods such as germiculture, IHC and PCR detection also needed to be used. Additionally, germiculture was time consuming and some tissue specimens were impossible to cultivate for diagnosis, therefore, bacterial detection could not be carried out owing to varied reasons. In this case, besides the general pathology special staining and molecular pathology PCR detection, clinical follow-up monitoring was also performed. The application of Auramine O staining, acid-fast staining and fluorescent quantitative PCR could provide powerful evidence for the pathological diagnosis of atypical *M. tuberculosis*. Due to the lower detection rate of *M. tuberculosis* using Auramine O and acid-fast staining, fluorescence quantitative PCR was commonly used to detect *M. tuberculosis* in tissues. The sensitivity and specificity of this method for detecting *M. tuberculosis* in sputum or surgical fresh specimens were 73.3% and 99.4%, respectively.[10]

However, the sensitivity and specificity of quantitative PCR for detecting mycobacteria in tissue specimens (65% and 85.3%, respectively) were slightly lower than those in sputum or surgical specimens. Also, the detection rate of *M. tuberculosis* using fluorescent quantitative PCR in formalin-fixed, paraffin-embedded histological sections of granulomatous lesions generally ranged from 50% to 60%.[3] In this study, the sensitivity and specificity of fluorescent quantitative PCR in the tissues retrieved with EDTA were 72% and 98.5%, respectively, which were significantly higher than the values obtained when PCR was performed directly after DNA extraction. Sometimes formalin-fixed, paraffin-embedded specimens of typical TB appeared in clinical pathological diagnosis, but fluorescence quantitative PCR could not detect *M. tuberculosis*. Some researchers believed that the DNA of tissues or bacteria in paraffin-embedded sections would be damaged to a certain degree. Targeted DNA of tissues could be ruptured and missed during the fixing process, which may lead to false-negative results.[9] Other researchers believed that it might be related to tissue protein cross-linking during formalin fixation.[6 7 10–13] High-temperature and high-pressure retrieval and EDTA heat-induced retrieval were the representative retrieval schemes for antigen retrieval in IHC, the principle of which is that the protein cross-linking induced by formaldehyde was broken by heating. So, the antigen sites were exposed, and contributed to the success of IHC.[14 15] This study attempted to introduce the method used in antigen retrieval into the process of fluorescent quantitative PCR detection, retrieved the fixed tissues,
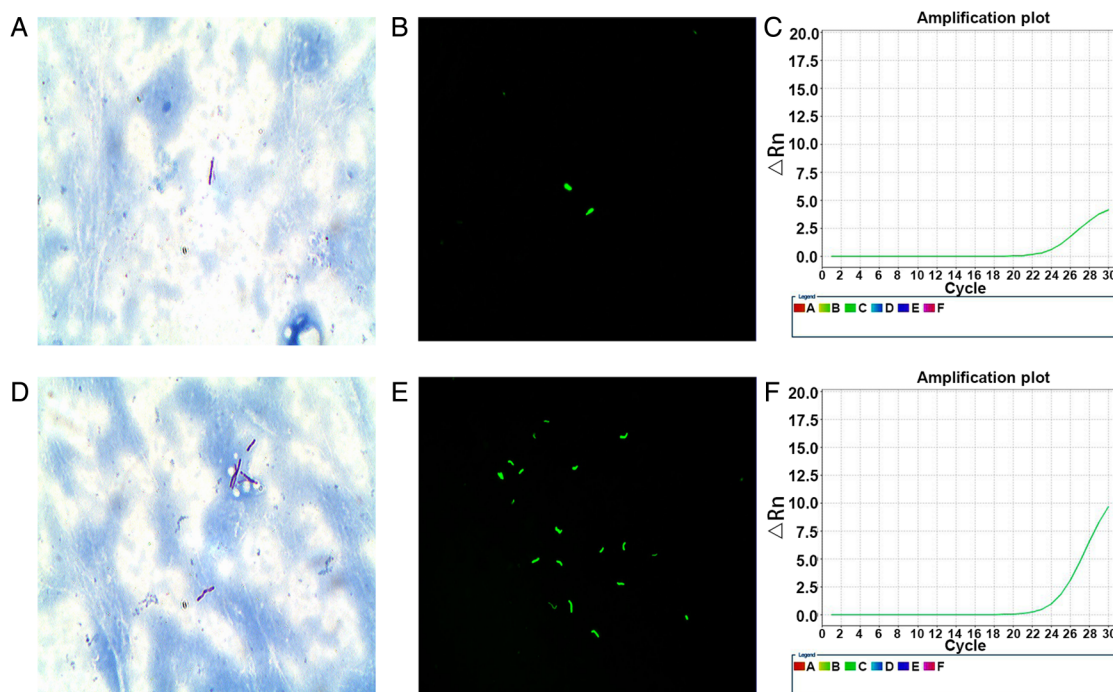
Figure 2 Fluorescent quantitative PCR and acid-fast and Auramine O staining of mycobacteria before and after EDTA retrieval. (A) Acid-fast stain before EDTA retrieval. (B) Auramine O stain before EDTA retrieval. (C) Fluorescence quantitative PCR amplification before EDTA retrieval. (D) Acid-fast staining after EDTA retrieval. (E) Auramine O staining after EDTA retrieval. (F) Fluorescence quantitative PCR amplification after EDTA retrieval.

eliminated the cross-linking between the tissue DNA and the bacterial DNA, digested the tissue using proteinase K, extracted DNA in tissues and hoped to improve the detection rate. Because EDTA heat-induced retrieval is suitable for the process of fluorescent quantitative PCR, the specimens were put into an EP tube to dewax, followed by EDTA heat-induced retrieval, DNA extraction and fluorescence quantitative PCR detection.

Fluorescent quantitative PCR showed that 75 of 125 (60%) specimens were positive for mycobacteria; this detection rate was slightly lower than the rate in sputum or surgical specimens. Some difference was found when the results of routine pathology special staining (46/125 (36.8%) for acid-fast staining and 61/125 (48.8%) for Auramine O staining) were compared; however, the detection rate was higher compared with the other two methods. Some cases were not detected by fluorescent quantitative PCR, but the results of acid-fast or Auramine O staining were positive, indicating that although the mycobacterial detection rate using routine fluorescent quantitative PCR in tissues was higher than the rate in the case of special staining (acid-fast and Auramine O staining), still false-negative results were obtained. The mycobacterial detection rate using fluorescent quantitative PCR after tissue retrieval with EDTA was 70.4% (88/125), and the positive cases were also completely detected in acid-fast and Auramine O staining, which indicated that the protein cross-linking caused by formalin could be broken by the pretreatment with EDTA, which contributed to tissue digestion and DNA extraction and avoided the false-negative results of fluorescence quantitative PCR detection. Comparing the Ct value of PCR amplification with or without EDTA retrieval indicated that the amplification efficiency was improved by EDTA retrieval. A statistically significant difference was found between the two groups ($p < 0.05$). Analysing the results of different types of specimens using fluorescence quantitative PCR detection revealed that the mycobacterial detection rate in different specimens increased after EDTA heat-induced retrieval. Due to the limition of quantity, It is not easy to make a pathological diagnosis for lung biopsy or bronchoscope biopsy specimens. Detection of mycobacterial in different specimens can be rapid and sensitive by fluorescence quantitative PCR after EDTA heat-induced retrieval, and false negative results can be avoided (table 3). These results indicated that the TB/non-tuberculous mycobacterial detection rate using fluorescent quantitative PCR could be increased after EDTA heat-induced retrieval. The mycobacterial detection rate increased by 10.4%, and the amplification efficiency was significantly improved (figure 2). Moreover, the specificity was high. The method was simple to carry out. Hence, further large-scale studies are required to validate the present findings before translating the research into clinical practice.

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Take home messages

► Fluorescent quantitative PCR with EDTA retrieval could greatly improve the detection rate of tuberculosis (TB)/non-tuberculous mycobacteria.
► EDTA retrieval contributed to avoid the false-negative results of fluorescence quantitative PCR detection of TB/non-tuberculous mycobacteria.
► EDTA retrieval improved the PCR amplification efficiency of fluorescent quantitative PCR detection of TB/non-tuberculous mycobacteria.

JCP

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