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Efficacy of real-time polymerase chain reaction for rapid diagnosis of endobronchial tuberculosis

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SUMMARY

Objectives: The definitive diagnosis of endobronchial tuberculosis (EBTB) is challenging because the disease manifests in various non-specific ways, and acid-fast bacilli (AFB) are often undetectable by sputum smear. The objective of this study was to evaluate the efficacy of real-time PCR of bronchoscopic biopsy specimens for the diagnosis of EBTB.
Methods: Real-time PCR amplification of *Mycobacterium tuberculosis* DNA in biopsy tissue from EBTB patients was performed prospectively. Diagnostic yields were compared for real-time PCR and for auramine O-stained sputum smears and bronchial brush smears. Whether diagnostic yield depended on bronchoscopic subtype of EBTB was also evaluated.
Results: Diagnostic yields were 4.1% (3/74) for sputum smear, 39.2% (29/74) for bronchial brush smear, and 89.2% (66/74) for real-time PCR. Real-time PCR melting curve analysis showed significantly higher yields than did AFB staining of bronchial brush smears for granular and caseating EBTB ($p < 0.01$).
Conclusions: Real-time PCR detection of *M. tuberculosis* DNA in EBTB biopsy tissue is more sensitive than sputum smear and bronchial brush smear, including at early disease stages. This PCR method may be a useful adjunct to culture- and smear-based techniques to allow more rapid EBTB diagnosis and timelier treatment.
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1. Introduction

Endobronchial tuberculosis (EBTB) is a less common and more serious form of tuberculosis infection. The definitive diagnosis is based on a positive stain for acid-fast bacilli (AFB) or the isolation of *Mycobacterium tuberculosis* from respiratory secretions combined with bronchoscopic examination.[1] Diagnosing EBTB is challenging because it manifests clinically and bronchoscopically in a variety of non-distinctive ways, sometimes mimicking the signs and symptoms of a range of diseases including asthma, lung cancer, pneumonia, and bronchiectasis.[2,3] In addition, patients with EBTB often have negative results on testing for the presence of AFB in sputum smears. The diagnostic yield is higher with endobronchial biopsy than with endobronchial brushing,[4,5] but it is still only 72.2%.[6] The identification of *M. tuberculosis* in cultured specimens can take 6–8 weeks even when specimens are in good condition.[7] Since a delayed EBTB diagnosis can allow the disease to progress to substantial fibrostenosis and other morbidities, even with occasional anti-tuberculosis chemotherapy,[2] early diagnosis is critical.

PCR allows the early diagnosis of tuberculosis with high sensitivity and specificity. However, PCR shows only 53–73% sensitivity in patients who are negative by sputum smear, although its specificity is 88–98%.[8,9] Real-time PCR has recently been described as a means to lower the risk of contamination associated with conventional PCR,[10] as well as increase speed, robustness, and reproducibility. However the diagnostic yield of real-time PCR for detecting *M. tuberculosis* in bronchoscopic biopsy tissue is unknown.

In the current study, we evaluated the diagnostic yields of real-time PCR for detecting *M. tuberculosis* DNA in bronchoscopic biopsy tissues in patients with different bronchoscopic subtypes of EBTB, and we compared those yields with yields from AFB-stained bronchoscopic brush smears.

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2. Methods

2.1. Subjects

This study was approved by the Institutional Ethics Review Board of The First Affiliated Hospital, China Medical University, Shenyang, China. A consecutive sample of patients at our hospital who had not undergone any previous anti-tuberculosis therapy and who were suspected of having EBTB were enrolled in the study between April 2011 and October 2012. All patients gave written informed consent prior to enrolment.

The diagnosis of EBTB was confirmed by positive bronchoscopic and histological examination and microbiological tests (tissue culture for tuberculosis and/or AFB stain of bronchial brushings) in accordance with the diagnostic criteria of the Chinese Thoracic Society.[11] As a control group, we enrolled consecutive patients with biopsy-confirmed lung cancer who were treated at our hospital during the same period.

2.2. Tissue collection and processing

Sputum was collected from all patients. One experienced pulmonologist used standard bronchoscopy (1T260, Olympus, Japan) to take two bronchial brushings from the bronchial segments where the EBTB lesion was located. Six bronchoscopic biopsies were also taken from the site of the tracheobronchial lesions, in the same segment; four were processed for histopathology, one for tissue culture, and one for real-time PCR.

Sputum smears and bronchoscopic brush smears were examined for the presence of AFB using the rapid auramine O fluorescent stain ('AO stain', Ourchem; Sinopharm Chemical Reagent Co. Ltd, Shanghai, China).[12] All biopsy samples were reviewed independently by two senior pathologists; discrepant conclusions were discussed until a consensus diagnosis was obtained. Based on bronchoscopic manifestations, the EBTB of each patient was classified into one of the following subtypes:[11] caseating, edematous–hyperemic, fibrostenotic, granular, tumorous, and ulcerative (Figure 1).

2.3. DNA isolation

DNA from biopsies was extracted with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The DNA concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific, USA).

2.4. Real-time PCR

Real-time PCR of bronchoscopic biopsies was performed by a technician blinded to the diagnosis. The following primer sets were used (Takara Bio, Dalian, China):[13] *M. tuberculosis* IS6110, 5′-TTGGAAAGGATGGGGTCA-3′ (forward) and 5′-CGCAGCCAACAC-CAAGTAG-3′ (reverse); and β-actin, 5′-AGTTGCCTTACACCCTT-TATTG-3′ (forward) and 5′-TCACCTTCACCGTTCCAGTTT-3′ (reverse). The *M. tuberculosis* primers gave an amplicon of 156 bp; the β-actin primers gave an amplicon of 149 bp. Screening experiments indicated that the optimal primer concentration was 0.4 μM (data not shown).

Real-time PCR was performed using the ABI PRISM 7500HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Thermal cycling conditions were as follows: initial denaturation at 95 °C for 1 min; amplification for 40 cycles of 95 °C for 5 s and 60 °C for 30 s; dissociation at 95 °C for 15 s, followed by extension at 60 °C for 1 min; and finally melting at 95 °C for 15 s. Each PCR reaction (20 μl) contained the following: SYBR Premix Ex Taq II (2×, 10 μl; Takara, Shiga, Japan), forward primer (10 μM, 0.8 μl), reverse primer (10 μM, 0.8 μl), ROX reference dye (50×, 0.4 μl; Takara, Shiga, Japan), DNA template (50 ng in 2.0 μl), and dH_2O (6.0 μl). Each reaction contained both primer pairs to amplify *M. tuberculosis* and β-actin DNA sequences as a control for DNA amplification. Each sample was tested in triplicate.

The cycle threshold (Ct) value was determined as a measure of the sensitivity of the RT-PCR assay. We compared the diagnostic yields based on fixed Ct values for which 35 and 40 were set as the cut-off values. If the annealing temperature of the melting curve generated using SDS software (version 2.0 a23; Applied Biosystems, Foster City, CA, USA) was 88.9 ± 0.1 °C, the amplification was judged to be positive and specific. All positive reactions were subjected to 2% agarose gel electrophoresis with a 100-bp DNA ladder

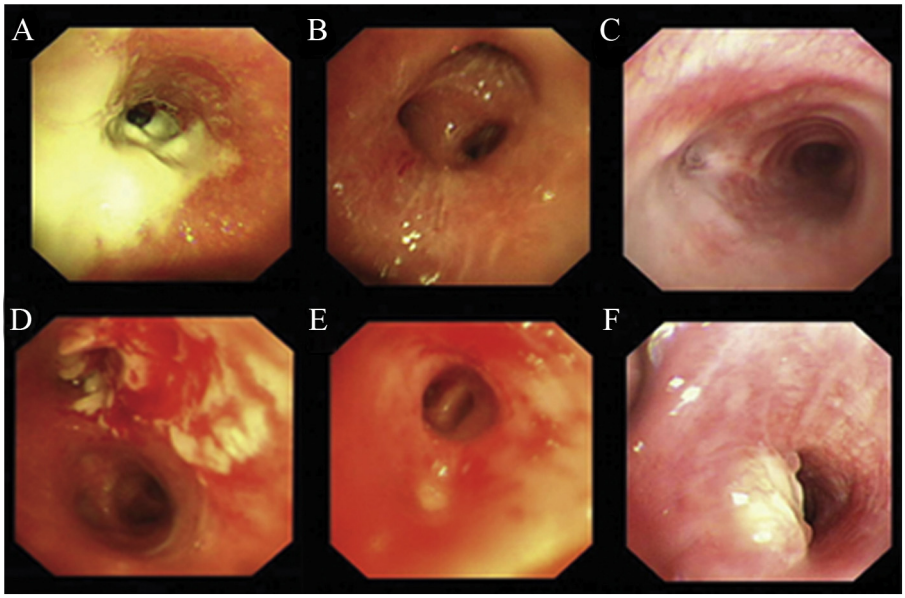

Figure 1. Representative bronchoscopy images from patients in our sample with different EBTB subtypes: (A) actively caseating, (B) edematous–hyperemic, (C) fibrostenotic, (D) granular, (E) ulcerative, and (F) tumorous.

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