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DIAGNOSTICS

Comparative evaluation of two rapid methods for differentiating mycobacteria

Xiaomao Yin [a], Lei Zheng [a], Lijuan Wu, Nannan Cao, Fen Zheng, Yanwei Hu, Min Lin, Peng Zhang, Qian Wang*

Laboratory Medical Center, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong Province 510515, PR China

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SUMMARY

The real-time PCR with duplex primer sets and the MPB64-based immunochromatographic assay are newly developed methods for rapid differentiation of mycobacteria. The aim of this study is to evaluate the two methods for differentiation between *Mycobacterium tuberculosis* complex and nontuberculous mycobacteria. A total of 95 clinical mycobacterial isolates belonging to 22 different species and 16 reference strains of 16 different species were differentiated by duplex real-time PCR method and MPB64-based immunochromatographic assay method. The two methods were evaluated by comparison with conventional biochemical technique as the gold standard method. The duplex real-time PCR method correctly differentiated all reference strains as well as the MPB64-based immunochromatographic assay method. For clinical isolates, the accuracy of the duplex real-time PCR method (100%) was slightly higher than the MPB64-based immunochromatographic assay method (97.9%), but there was no statistical significance between the two methods ($P > 0.05$), and there was an excellent agreement between them (*Kappa* = 0.957). The duplex real-time PCR method possesses greater potential for differentiation of mycobacteria in the clinical laboratory than the MPB64-based immunochromatographic assay method. However, the MPB64-based immunochromatographic assay method is more convenient than the duplex real-time PCR method when the number of sample is small.

1. Introduction

The genus *Mycobacterium* comprising of a wide range of species is generally classified into two subgroups, namely *Mycobacterium tuberculosis* complex (MTC) and nontuberculous mycobacteria (NTM). MTC consists of *M. tuberculosis*, *Mycobacterium bovis*, *Mycobacterium canetti*, *Mycobacterium africanum*, and *Mycobacterium microti*. The complex could cause animal or human TB which is different from infection caused by NTM in pathogenesis and treatment regimen. Therefore, the ability to identify and differentiate MTC from NTM is of importance for patient management, hospital control of infection, and public health TB control services.[1] However, the traditional differentiation method which recognizes phenotypic and biochemical characteristics is slow, cumbersome and time-consuming.[2] In order to overcome these disadvantages, several rapid diagnostic techniques for identification of mycobacteria have been developed. Of these new technical strategies, gas–liquid chromatography and high-performance liquid chromatography differentiate species by recognizing fatty acids and mycolic acids respectively, and most of molecular biology tests for identification of mycobacteria are based on the combination of nucleic acid amplification and hybridization with specific oligonucleotide probe.[3] Although these methods are technically excellent and time-saving, they are costly and laborious.

Recently, a real-time PCR assay using duplex primer sets and melt curve analysis has been developed for differentiating MTC from NTM.[4] The assay adopts *rpoB* gene of mycobacteria as the amplified target and utilize SYBR green dye for melt curve analysis. Unlike some previous real-time PCR techniques,[5–14] this assay does not need additional probes for hybridization. Moreover, it could generate amplification products not only for MTC but also for NTM; some similar real-time PCR methods using duplex or multiplex primer sets only generate PCR fragments for either MTC or NTM.[15] So it is necessary to add control primer set in the PCR reaction system for those methods but not for this assay. The two primer sets used in this assay specifically amplify a 235-bp DNA sequence from MTC and a 136-bp DNA sequence from NTM respectively.[16]

* Corresponding author. Tel./fax: +86 20 61641007.
E-mail addresses: nfyyWangQian@163.com, lunwentongxun@163.com (Q. Wang).
[a] Xiaomao Yin and Lei Zheng contributed to this study equally.

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A positive MTC identification and a positive NTM identification are both defined as amplification channel with the normal fluorescence threshold set at 0.5, but the melt curve peaks of them are at 90–91 °C and at 88–90 °C respectively. Combined with bead extraction method, a differentiation between MTC and NTM can be achieved in 2.5 h with great accuracy.[4]

Another simple, rapid and low-cost test for differentiation of mycobacteria, the Capilia TB assay (TAUNS, Numazu, Japan), was developed prior to the real-time PCR method above-mentioned and are commercially available in the world.[17,18] Target antigen of the immunochromatographic assay (ICA) is MPB64, a highly specific secreted protein for MTC and also described as MPT64. The MPB64-ICA slide test kit consists of a plastic slide with a sample pad, a reagent pad, a nitrocellulose membrane and an absorbent pad. Two monoclonal anti-MPB64 antibodies, one of which has been conjugated with colloidal gold particles, are used for antigen capture and detection in a sandwich-type assay. In presence of MPB64, the anti-MPB64 monoclonal antibody conjugated with colloidal gold particles binds to the antigen and then the primary complex further binds to another antibody. Finally the immobilized antibody-antigen-gold conjugate antibody complex forms and gives a color band.[17] The results are readable within 15 min and there is no need for any special equipment.

So far the performance of the duplex primers real-time PCR assay compared with the commercial Capilia TB assay has not been evaluated. Therefore, the aim of this study was to perform a comparative study to assess the ability of both tests for differentiation between MTC and NTM.

2. Materials and methods

2.1. Mycobacterial strains

Reference strains of 16 *Mycobacterium* species were included in this study: *Mycobacterium abscessus* ATCC 19977, *Mycobacterium aurum* ATCC 23366, *Mycobacterium avium* ATCC 25921, *Mycobacterium chelonae* ATCC 35752, *Mycobacterium fortuitum* ATCC 6841, *Mycobacterium gordonae* ATCC 14470, *Mycobacterium intracellulare* ATCC 13950, *Mycobacterium kansasii* ATCC 12478, *Mycobacterium neoaurum* ATCC 25795, *Mycobacterium nonchromogenicum* ATCC 19530, *Mycobacterium parafortuitum* ATCC 19686, *Mycobacterium scrofulaceum* ATCC 19981, *Mycobacterium smegmatis* ATCC 19420, *Mycobacterium triviale* ATCC 23292, *M. bovis* ATCC 19210, *M. tuberculosis H37Rv*. This study did not involve human or animal subjects.

A total of 95 clinical mycobacterial isolates belonging to 22 different species were used in this study: 6 *M. abscessus*, 1 *Mycobacterium asiaticum*, 3 *M. avium*, 5 *M. chelonae*, 1 *M. fortuitum*, 2 *M. gordonae*, 1 *Mycobacterium hemophilum*, 3 *M. intracellulare*, 3 *M. kansasii*, 1 *Mycobacterium malmoense*, 1 *Mycobacterium marinum*, 1 *M. nonchromogenicum*, 1 *M. scrofulaceum*, 1 *Mycobacterium shimodei*, 2 *M. smegmatis*, 2 *Mycobacterium simiae*, 3 M. *szulgai*, 1 *Mycobacterium terrae*, 1 *M. triviale*, 1 *Mycobacterium ulcerans*, 5 *M. bovis*, 50 *M. tuberculosis*.

Reference strains were cultivated in Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose complex plus 0.05% Tween 80 and confirmed by Zeihl–Neelsen staining after 3 weeks. Clinical mycobacterial strains were isolated from sputum, bronchoalveolar lavage fluid, cerebrospinal fluid, pleural fluid or urine. All these specimens were acquired from different patients hospitalized in Guangzhou Chest Hospital with mycobacterial disease and were processed by the *N*-acetyl-L-cysteine (NALC)-NaOH method with slight modification. The processed specimens were inoculated onto Lowenstein–Jensen slants and cultured for several weeks. All clinical mycobacterial isolates were identified by conventional biochemical technique and confirmed by analysis of 16S–23S internal transcribed spacer sequence.

2.2. MPB64-ICA test

About 100 μl sample from liquid medium was applied to the MPB64-ICA slide test directly without any manipulation. For solid medium, a loopful of bacterial colonies from the surface of Lowenstein–Jensen slant was suspended in sterilized water. Then the suspension was homogenized and adjusted to a McFarland turbidity standard of 1.0. Approximately 100 μl of bacterial suspension was applied into the sample well of the plastic slide and the results would be observed after 15 min.

The presence or absence of a color band in the test zone was recorded as "positive (+)" or "negative (−)" respectively only when there was also a color band in the control zone. If the MPB64-ICA result of mycobacterial strain was positive, this strain was differentiated as MTC. In turn, this strain was defined as NTM if the MPB64-ICA result was negative.

2.3. DNA extraction

A loopful of bacterial colonies was scraped from Lowenstein-Jensen slant and suspended in sterilized water. The suspension was homogenized by a vortexer and adjusted to a Mcfarland turbidity standard of 1.0 by a nephelometer. Then 1 ml of suspension was transferred to 1.5-ml microcentrifuge tube and centrifuged at 10,000 × g for 5 min. The supernatant was poured off and the sediment was resuspended in 40 μl DNA extraction solution (PG Biotech Company, Shenzhen, China) by vortex. The resuspended pellet was subsequently heated at 100 °C for 15 min and centrifuged at 13,000 × g for 10 min after cooled. Finally, the supernatant containing the extracted bacterial nucleic acids was transferred to another 1.5-ml tube and preserved at −20 °C until use.

2.4. Real-time PCR assay with duplex primer sets

Two primer sets described previously was used for real-time PCR assay.[16] Primer set Tbc 1 and TbcR 5 was used to amplify MTC specifically and primer set M5 and RM3 was used for specific amplification of NTM. The 20 μl reaction mixture contained 10 μl MightyAmp for Real Time (SYBR Plus) (Takara Bio, Dalian China), 0.4 μl ROX Reference DyeII, 0.1 μl of 50 μM each PCR primer, 8.2 μl ddH_2O and 1 μl DNA template. The real-time PCR was performed using an Applied Biosystems 7500 fast Real-Time PCR System (Applied Bio, Foster City, USA) and consisted of 98 °C for 2 min, 30 cycles of 98 °C for 10 s and 65 °C for 15 s, with a final extension at 68 °C for 30 s. Post-amplification melt curves were generated and analyzed using Applied Biosystems 7500 fast Real-Time PCR System software v 2.0 (Applied Bio, Foster City, USA).

2.5. Mutation detection of mpb64 gene

Primer set T1 and T6 described previously was used to amplify the *mpb64* gene of clinical MTC isolate for which the result of MPB64-ICA was negative.[19] The basic 50 μl amplification reaction mixture containing 2.0 μl of extracted DNA, 1.25U of *Taq* DNA polymerase (Takara Bio, Dalian China), 0.5 μl of 50 μM each PCR primer, 5 μl of 10 × *Taq* PCR buffer (Takara Bio, Dalian China) and 4 μl of 2.5 mM each deoxynucleotide triphosphate was used for amplification of *mpt64* gene with a Mastercycler ep gradient thermocycler (Eppendorf, hamburg, Germany). The amplification profile was initial denaturation at 94 °C for 2 min, then 30 cycles of