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DIAGNOSTICS

Direct identification and discernment of *Mycobacterium avium* and *Mycobacterium intracellulare* using a real-time RNA isothermal amplification and detection method

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SUMMARY

The purpose of this work was to establish a real-time simultaneous amplification and testing method for identification and discernment of *Mycobacterium avium and Mycobacterium intracellulare* (SAT-MAC assay) and to evaluate the efficiency with which this method can detect isolated strains and clinical sputum specimens. The specific 16S rRNA sequences of *M. avium* and *M. intracellulare* were used as targets to design RNA probes and a reverse transcription primer containing T7 promoter. RNA isothermal amplification and real-time fluorescence detection were performed at 42 °C. SAT-MAC assay, culture tests on Lowenstein-Jensen (L-J) culture medium and PCR-sequencing were used to test the clinical isolated strains and sputum specimens. The limit of detection (LOD) of *M. avium* and *M. intracellulare* by SAT-MAC was found to be 30 CFU/mL and 20 CFU/mL. SAT-MAC showed high specificity in 21 species of mycobacteria standard strains and 5 species of non-mycobacteria bacteria. Using PCR-sequencing as the reference method, both rates of SAT-MAC assay for identifying *M. avium* and *M. intracellulare* from clinical isolates were 100% (259/259). Consistent with the results of L-J culture combined PCR-sequencing, the coincidence rate of SAT-MAC assay in clinical sputum specimens was 100% (369/369) for *M. avium* and 99.19% (366/369) for *Mycobacterium intracellular.* The SAT-MAC assay can identify and distinguish *M. avium* and *M. intracellulare* rapidly and accurately. It may be suitable for use in clinical microbiology laboratories.

1. Introduction

Mycobacterium avium and *Mycobacterium intracellulare* are important pathogens in *M. avium-intracellulare complex* (MAC) [1,2]. They can invade the lungs, lymph nodes, bones, joints, skin, and soft tissue and can spread themselves systemically in humans and other animals [3–5]. MAC infections may result in severe disease or death if left untreated or treated improperly [6], especially among immunocompromised individuals. The MAC infection rate among HIV patients is roughly 30–80% [6]. Additionally, patients infected by MAC present clinical symptoms similar those of tuberculosis (TB) patients but require different medical treatment. MACs have been found to be resistant to most drugs used to treat TB [7]. Although *M. avium* and *M. intracellulare* are both MACs, they differ in the hosts infected [4,8], and in susceptibility to antibiotic drugs [9]. For these reasons, distinguishing and identifying *M. avium* and *M. intracellulare* is of considerable significance in clinical diagnosis and treatment of these infections. MAC is slow-growing mycobacteria with a long growth cycle, so traditional methods of biochemical identification methods take a long time [10,11]. In the present study, a real-time simultaneous RNA isothermal amplification and detection method for the detection of *M. avium* and *M. intracellulare* (SAT-MAC) was established and its use in testing clinical isolates and sputum specimens was evaluated.

2. Materials and methods

2.1. Strain collection and processing

The 21 Mycobacterium reference strains were provided by the National Tuberculosis Reference Laboratory (Beijing, China). The

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reference strains were *Mycobacterium tuberculosis* standard strain $H_{37}Rv$ (ATCC 27294), *Mycobacterium kansassi* (ATCC 12478), *M. intracellulare* (ATCC 13950), *Mycobacterium chelonae* (ATCC 14472), *Mycobacterium phlei* (ATCC 11758), *Mycobacterium fortuitum* (ATCC 6481), *Mycobacterium gordonae* (ATCC 14470), *Mycobacterium aurum* (ATCC 23366), *Mycobacterium gilvum* (ATCC 43909), *Mycobacterium parafortuitum* (ATCC 19686), *Mycobacterium smegmatis* (ATCC 19420), *Mycobacterium marinum* (ATCC 927), *Mycobacterium aichiense* (ATCC 27280), *Mycobacterium neoaurum* (ATCC 25795), *Mycobacterium terra* (ATCC 19619), *Mycobacterium nonchromogenicum* (ATCC 19530), *Mycobacterium vaccae* (ATCC 15483), *Mycobacterium microti* (ATCC 19422), *M. avium* (ATCC 25291), *Mycobacterium scrofulaceum* (ATCC 19981), and *Mycobacterium malmoense* (ATCC 29571). Five strains of pathogenic non-mycobacteria commonly seen in the respiratory tract were provided by the clinical laboratory of Shanghai Pulmonary Hospital. These were *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Hemolytic streptococcus*, *Pseudomonas pyocyaneum*, and *Acinetobacter baumannii*. We obtained 216 clinical nontuberculous mycobacteria (NTM) strains and 43 *M. tuberculosis* (MTB) strains from Shanghai Key Laboratory of Tuberculosis. All the clinical strains were isolated from different patients with pulmonary diseases. All mycobacteria were cultured using Middlebrook 7H10 agar culture medium supplemented with oleic-albumin-dextrose-catalase (OADC) (Becton, Dickinson, NJ, U.S.). Positive cultures of all strains were collected during the logarithmic phase, and saline was added until the bacterial turbidity reached 1 mg/mL (approximately 10^7 CFU/mL) tested by BD PhoenixSpec Nephelometer (Becton, Dickinson, NJ, U.S.). Then 1 mL bacterial suspension was used for SAT-MAC assay, 1 mL for PCR-sequencing, and 1 mL for re-test as a backup.

2.2. Collection and processing of clinical specimens

Here, 322 sputum specimens from suspected pulmonary tuberculosis patients (including 209 smear-positive and 113 smear-negative sputum specimens) and 47 sputum specimens (smear-negative) from patients with other respiratory diseases were collected. All specimens were collected from inpatients from Shanghai Pulmonary Hospital from May to July 2013. Each approximately 5 mL specimen was decontaminated using the N-acetyl L-cysteine (NALC)-NaOH method [12]. The processed sediment was washed once using PBS (PH 7.6) sterile solution, resuspended in 1 mL PBS sterile solution, and separated in 100 μL aliquots in 1.5 mL tubes for SAT-MAC, PCR-sequencing, and Lowenstein-Jensen (L-J) culture medium culture tests.

2.3. SAT-MAC assay

The specific sequences of 16S rRNA of *M. avium* and *M. intracellulare* were used as targets to design RNA probes and reverse transcribed by M-MLV reverse transcriptase to generate a 108 bp cDNA fragment using a pair of specific primers incorporating the T7 promoter in the antisense primer. The DNA was then transcribed to RNA using T7 RNA polymerase and then subjected to successive cycles of amplification. An internally labeled probe that releases a fluorescent signal when hybridized with the target RNA was used. Kinetic measurement of the real-time amplification fluorescent signal was detected using a real-time PCR instrument. *M. avium* and *M. intracellulare* were identified in separate tubes using the same primers 5′-CTGGGAAACTGGGTCTAATA-3′ and 5′-AATTTAATACGACTCACTATAGGGAGACCACCAACAAGCTGATAGGC-3′. The probe sequences of the *M. avium* and *M. intracellulare* were 5′-CGUGGAUAGGACCUCAAGACGCCACG-3′ and 5′- CGGUGACCUUUAGGCGCACCG-3′, respectively, labeled with 6-carboxyfluorescein (FAM) phosphoramidite at the 5′ end and with 4-[4-(dimethylamino) phenylazo] benzoic acid *N*-succinimidylester (DABCYL) at the 3′ end. Primers and RNA probes were synthesized by Thermo Fisher Scientific Inc (Shanghai, China) and Shanghai GenePharma Co, Ltd (Shanghai, China), respectively.

All samples were centrifuged at 13,000 × *g* for 5 min and the supernatant was discarded. Then 50 μL of sample dilution solution (10 mM sodium citrate, pH 8.0) was added for re-suspension and vortexed. Each sample was sonicated for 15 min at room temperature in a water bath sonicator (Shanghai Sheng-yan Ultrasound Machines Co. Ltd, Shanghai, China) at 300 W and centrifuged. The supernatant served as a template in SAT-MAC assay. The live *M. avium* (ATCC25291) and *M. intracellulare* (ATCC13950) were used as positive controls and double distilled water served as a negative control.

The real-time simultaneous RNA isothermal amplification and detection method were performed as described by Cui [13]. Briefly, 2 μL processed supernatant and 30 μL reaction solution (40 mM Tris−HCl buffer pH 8.1, 8 mM magnesium chloride, 25 mM sodium chloride, 2 mM spermidine, 5 mM dithiothreitol, 80 μg/mL bovine serum albumin, 200 μM dATP, dTTP, dGTP, and dCTP, and 1 mM ATP, GTP, CTP, and UTP; upstream and downstream primers and probes at final concentrations of 0.5 mmol/L) were prepared in a 200 μL PCR tube. The mixture was pre-incubated at 60 °C for 10 min, followed by 42 °C for 5 min, then a 10 μL aliquot containing 2000 units M-MLV reverse transcriptase and 2000 units T7 RNA polymerase was added (RD Bioscience, Inc., San Diego, CA, U.S.). The mixture was gently mixed, and the reaction was placed immediately into a 7500 real-time PCR system (Applied Biosystems Inc., Foster City, CA, U.S.). RNA isothermal amplification was conducted at 42 °C for 1 min, with a total of 40 cycles. FAM fluorescence data were collected after each cycle. Specimens with cycle thresholds (C_t) less than or equal to 40 were classified as positive. These processes were performed in a biological safety cabinet class II B2 in a bio-safe PII laboratory. The RNase-free tubes and tips were needed for the SAT-MAC assay. After the reaction was complete, the reaction tube was immediately soaked in 2000 mg/mL sodium hypochlorite solution, and 1000 mg/mL sodium hypochlorite solution was used to clean the work area and tools.

2.4. Determination of SAT-MAC specificity

Positive-culture microorganisms of 21 standard strains of mycobacterium and 5 non-mycobacteria bacteria were collected and diluted to turbidity 1 mg/mL using saline; 1 mL bacterial suspension was used for the SAT-MAC assay in order to evaluate its specificity.

2.5. Determination of limit of detection (LOD) of SAT-MAC

M. avium and *M. intracellulare* in log-phase growth was adjusted to McFarland No. 1 (approximately 10^7 CFU/mL) in sterile saline, and then further diluted with progressive 10-fold dilutions to 10^{-10} CFU/mL in tubes with glass beads containing 9 mL sterile saline. A 1 mL aliquot from each dilution was centrifuged in 1.5 mL tubes and the sediments were processed for SAT-MAC assay as described above. A 0.1 mL aliquot from each dilution was inoculated into one Middlebrook 7H10 agar culture medium tube for the colony count of *M. avium* and *M. intracellulare*. The average number of colonies corresponding to the lowest concentration that could be detected by SAT-MAC assay was considered indicative of the LOD of SAT-MAC assay.

A pooled sputum sample was prepared from 10 patients with non-mycobacterial respiratory disease and divided into ten equal sputum specimens [14]. A 1 mL aliquot from each dilution was spiked in a corresponding sputum specimen and vortexed. The sputum specimens were processed for SAT-MAC as described

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