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Original Article

Evaluation of a real-time PCR assay for detection of *M. avium* strains

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Abstract: The emerging prevalence of non tuberculous Mycobacterium species (NTMs) is especially that of *M. avium* complex is becoming a matter of growing concern in countries where a low TB incidence is reported. In our study, MGB probes and specific primers were designed according to IS1245 gene of *M. avium*, and the sensitive, specific and rapid real-time PCR assay for *M. avium* was established, and was used to detect *M. avium* in simulation samples. The minimum detectable concentration is 10^1 copies for M. avium DNA. The standard curve showed correlation coefficient between threshold cycle and IS1245 gene fragment copy number was 0.994 and slope is -3.735, which showed a good linear relationship. In addition, the minimum detectable concentration is 10^2 cells/ml for simulation sample. Also, the other nine strains showed negative results by the assay, which proved good specificity. This assay had high sensitivity and specificity for identification of M. avium from the simulation specimens. The established real-time PCR should be useful for ecological and epidemiological surveillance of M. avium strains.

Keywords: Real-time PCR, *M. avium*

Introduction

The emerging prevalence of non tuberculous Mycobacterium species (NTMs) especially that of *M. avium* complex is becoming a matter of growing concern in countries where a low TB incidence is reported [1, 2]. Respiratory infection in humans by NTM is primarily caused by M. avium complex [3]. Nowadays, the incidence of M. tuberculosis (MTB) and NTMs like M. avium and M. kansasii has increased due to Human Immunodeficiency Virus (HIV) epidemic [4]. The commonest NTM's associated with pulmonary infection among HIV patients are M. avium complex, M. kansasii, M. abscessus and M. fortuitum [5]. A number of tools have been used in TB diagnosis, for instance smear microscopy the most widely used test has low sensitivity especially in patients with extra pulmonary tuberculosis, those with HIV co-infection and TB due to NTMs [6]. Nucleic acid amplification tests such as PCR based assay have great promise for TB diagnosis and rapid detection of drug resistance with commercial assays widely used in developed countries for over 20 years [7]. However despite their simplicity, they are prone to PCR inhibitors, some tests require post amplification procedures that increases the turn-around time and some are limited by the DNA quantities in the starting material [8, 9]. Furthermore, since most of these techniques require the isolates to be cultured first, this will introduce growth competition in cases of mixed infection and hence a selection bias. Therefore accurate, rapid and cost effective methods for the identification of these NTMs and MTB are greatly needed for appropriate TB management.

Rapid, specific, and sensitive methods for the detection and identification of mycobacteriosis are needed to avoid unnecessary delay in making appropriate decisions.

IS1245 is a 1,313-bp element delimited by two imperfect inverted repeats with an open read-

Table 1. Strains used to confirm the specificity of the assay

No.	Strains
H37Rv	*M. tuberculosis*
95055	*M. bovis*
95016	*M. asiaticum*
95017	*M. sgrofulaeeum*
95015	*M. simiae*
95147	*M. kansasii*
95018	*M. gordonae*
95022	*M. fortuitum*
95021	*M. chelonae subsp. abscessus*

Table 2. Probe and Primers for real-time PCR of *M. avium* Targets

Prob/Primer	Sequence (5'-3')
Fwd primer	CGGGGAGTGGTGTAAGTGATG
Rev primer	CTCGGTAGTGATTCTTGGTCGTT
Probe	FAM-CCACAGAGACTCACGC-MGB

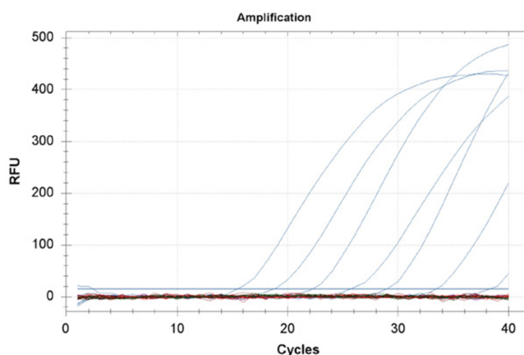

Figure 1. Sensitivity plots of primers and probes used in real-time PCR amplification of IS1245 gene of *M. avium*, from 10^7 to 10^0 copies.

Table 3. Reproducibility and intra-assay coefficient of variation for the real-time PCR amplification in study

Dilution	Ct value			CV (%)
10^7	14.86	15.14	15.44	1.91
10^6	18.88	19.08	18.76	0.86
10^5	22.23	21.89	22.25	0.91
10^4	25.93	25.66	25.69	0.57
10^3	28.85	28.33	28.98	1.20
10^2	33.03	32.74	33.43	1.05
10^1	36.30	37.00	38.27	2.69
10^0	N	N	N	N

Note: N, Detected negative.

Table 4. Reproducibility and inter-assay coefficient of variation for the real-time PCR amplification in study

Dilution	Ct value			CV (%)
10^7	15.44	14.35	16.71	7.62
10^6	18.76	19.27	22.21	9.27
10^5	22.25	22.25	24.25	5.04
10^4	25.69	25.37	26.96	3.23
10^3	28.98	29.02	29.58	1.15
10^2	33.43	34.73	37.03	5.20
10^1	38.27	37.09	39.54	3.20
10^0	N	N	N	N

Note: N, Detected negative.

ing frame encoding a putative transposase. The presence of multiple copies of IS1245 in all human M. avium clinical isolates tested prompted us to investigate the use of this element for epidemiological purposes, much as has been done with IS6110 from M. tuberculosis [10]. RFLP using IS1245 appeared to have a discriminatory power for strain differentiation comparable to that achieved with PFGE [11].

In our study, MGB probes and specific primers were designed according to IS1245 gene of *M. avium*, and the sensitive, specific and rapid real-time PCR assay for *M. avium* was established, and was used to detect *M. avium* in simulation samples.

Materials and methods

DNA preparation of M. avium strains

We used a *M. avium* strain 95001 purchased from ATCC to establish the real-time PCR method and prepare the simulation samples. The genomic DNAs of reference strains used in the experiment including 34 *M. avium* strains from clinic and nine other strains (H37Rv and eight NTM strains, see **Table 1**), were provided by the corresponding laboratory of National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention (China CDC).

The strains were cultured using a standard Löwenstein-Jensen medium method, heat inactivated and then used directly in polymerase chain reactions (PCRs).

Preparation of simulation samples

The simulated sputum contained 47 mg/ml Mucin, 6 mg/ml Salmon sperm DNA, 3.6 mg/

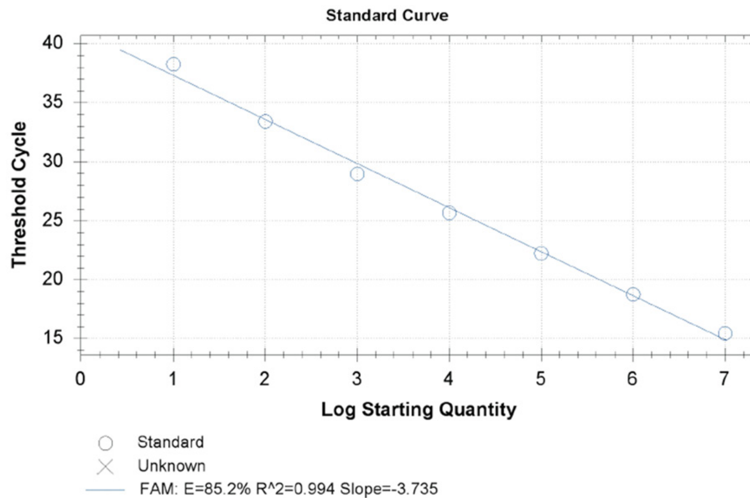

Figure 2. Regression plots of primers and probes used in real-time PCR amplification of IS1245 gene fragment of *M. avium*.

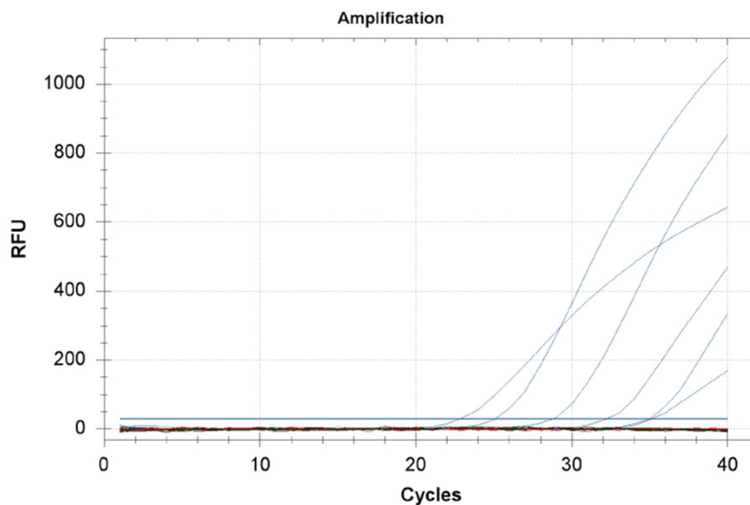

Figure 3. Sensitivity plots of primers and probes used in real-time PCR amplification of simulation samples of *M. avium*, from 10^7 to 10^0 cells/ml.

ml phosphatidylcholine, 33 mg/ml Bovine Serum Albumin and 116 mM NaCl. The inocula were prepared from actively growing bacteria collected from Lowenstein-Jensen slants or 7H10 agar plates. The inocula were adjusted with saline to a cell density of 3 × 10^8 cells/ml (McFarland 1 standard). Then we diluted the inocula by the simulated sputum from 10^7 to 10^0 cells/ml.

Quality control

Reagents were aliquoted and each aliquot was used only once. Sterile microfuge tubes and 96 PCR well plates for Real time PCR assay use. Reagent preparation, DNA extraction, DNA amplification and detection were performed in separate rooms to avoid cross contamination of amplicons.

Real-time PCR primers and probes

We design the primers and probe by ABI primer Express 2.0 software (synthesized by Shang-hai GeneCore BioTechnologies Co.,Ltd.), according to the conserved sequences of *M. avium* provided by Gene-Bank (*M. avium* -gb|CP008-744.1|), see **Table 2**. The primers were also used to prepare the standard plasmid.

Construction of standard plasmid

To determine the sensitivity of the real-time PCR method, a recombinant plasmid containing the target sequence of *M. avium IS1245* gene from ATCC strain 95001 (*M. avium*) was constructed as follows.

DNA was amplified with primers from 95001 strain, the fragment contains 71 bp probe target sequences. The PCR products (87 bp) were cloned into the pMD-19T vector using the pMD-19T vector Cloning Kit (Takara, Dalian, China). The recombinant *Escherichia coli* strain DH-5α carrying the recombinant plasmid was inoculated on LB solid culture medium (including Ampicillin penicillin) and inoculated at 37°C overnight. The recombinant plasmid DNA was extracted with a TIANpure Midi Plasmid Kit (TIANGEN, China) and validated by sequencing. The recombinant plasmidwas quantified by NanoDrop® Spectrophotometer ND-1000 (IMPLEN, Germany) at 260nm and was serially diluted (concentration range $1.0 \times 10^7 \sim 1.0 \times 10^0$ copies/μl) to evaluate the limit of detection of the real-time PCR method.

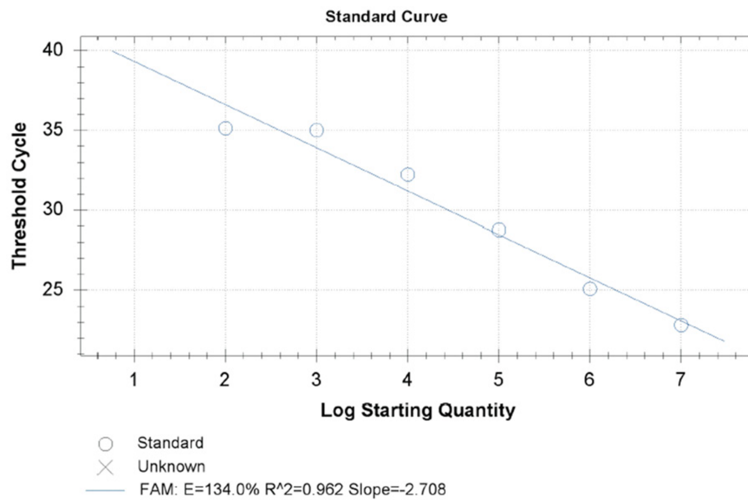

Figure 4. Regression plots of primers and probes used in real-time PCR amplification of IS1245 gene fragment of *M. avium* in simulation samples.

Table 5. Amplification of IS1245 gene fragment of *M. avium* in simulation samples

Dilution	10^7	10^6	10^5	10^4	10^3	10^2	10^1	10^0
Ct value	22.81	25.10	28.78	32.23	35.01	35.13	N	N

Real-time PCR reaction conditions

Fluorescence quantitative PCR instrument is BIO RAD MJ MiniOpticon, 20 μl reaction system including: 10 μl 2× Premix Ex Taq (TaKaRa DRR390), 250 nmol/L primers, 250 nmol/L probe, 6.5 μl deionized water, and 10^7~10^0 copies/μl, each 2 μl, DNA templates with different concentrations. The reaction procedures: initial denaturation 95°C 10 min, then 40 cycles, 95°C 15 s, 55°C 60 s. Each operation uses three parallel samples intra-assay.

Results

Standard curves and sensitivity

The detection range is 10^1~10^7 copies/μl by the established assay in this study, minimum detectable concentration is 10^1 copies (**Figure 1** and **Table 4**). The standard curve showed correlation coefficient between threshold cycle and IS1245 gene fragment copy number was 0.994 and slope is -3.735 (**Figure 2**).

Repeatability

Reproducible experimental results show that the range of intra-assay coefficient of variation is 0.57%~2.69% (**Table 3**) and the range of inter-assay coefficient of variation in is 1.15%~9.27% in each standard concentration point (**Table 4**).

Specificity

The results were positive for 34 clinic *M. avium* strains in our test, and the others results were all negative for other nine strains (**Table 1**).

Detection of simulation samples

The detection range is 10^2~10^7 cells/ml by the established assay in this study and the minimum detectable concentration is 10^2 cells/ml. The standard curve showed correlation coefficient between threshold cycle and IS1245 gene fragment copy number was 0.962 and slope is -2.708 (**Figures 3**, **4** and **Table 5**).

Discussion

The genus Mycobacterium encompasses a large number of worldwide distributed pathogenic and opportunistic bacteria responsible for a broad range of infectious disease. *Mycobacterium tuberculosis* complex, *Mycobacterium avium*, and many other nontuberculous mycobacteria (NTM) are of major medical and veterinary significance [12]. With increased incidence of TB and non tuberculous disease infection especially among HIV patients, diagnostics with better sensitivity and ability to identify M. tuberculosis and non tuberculous mycobacteria are required for appropriate management.

We chose a fragment of specific gene IS1245 for M. avium as target used in the qPCR. In this study, we established a rapid, sensitive and specific qPCR for detection of M. avium. The minimum detectable concentration is 10^1 copies for M. avium DNA. The standard curve showed correlation coefficient between threshold cycle and IS1245 gene fragment copy number was 0.994 and slope is 3.735, which showed a good linear relationship. In addition, the minimum detectable concentration is 10^2 cells/ml for simulation samples. Also, the other

nine strains showed negative results by the assay, which proved good specificity.

The minimum detectable concentration of M. avium DNA is higher than that of simulation samples. It is understandable as there are some inhibitors existed in simulation samples. The correlation coefficient of DNA is also higher than that of simulation samples, also owning to inhibitors.

The limitation of this study is that we do not apply the assay to clinic samples. However, the assay is used to detect simulation samples of M. avium and the minimum detectable concentration reached to 10^2 cells/ml. The assay is perspective for detection of M. avium in clinic samples.

In conclusion, this assay had high sensitivity and specificity for identification of M. avium from the simulation specimens. The established real-time PCR should be useful for ecological and epidemiological surveillance of *M. avium* strains.

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Disclosure of conflict of interest

None.

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