Journal of Molecular Diagnostics, Vol. 11, No. 1, January 2009

DOI: 10.2353/jmoldx.2009.080081

# Multi-Probe Real-Time PCR Identification of Common *Mycobacterium* Species in Blood Culture Broth

Suporn Foongladda,* Suporn Pholwat,* Boonchuay Eampokalap,† Pattarachai Kiratisin,* and Ruengpung Sutthent*

*From the Department of Microbiology,* Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok; and the Microbiology Laboratory,† Bamrasnaradura Institute, Ministry of Public Health, Nonthaburi, Thailand*

***Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* are the most common causes of systemic bacterial infection in AIDS patients. To identify these mycobacterial isolates in primary blood culture broths, we developed a multiple hybridization probe-based real-time PCR assay using the LightCycler system. The primers were designed to amplify a 320-bp fragment of *Mycobacterium* 16S rRNA genes. Reaction specificity was evaluated using PCR amplification curves along with specific melting temperatures of probes on DNA extracted from 13 *Mycobacterium* species. In this study, results showed 100% accuracy for the selected bacterial panel. Detection limits were 350, 600, and 650 colony-forming unit (CFU)/ml blood culture broths for *M. tuberculosis* complex, *M. avium*, and *M. intracellulare*, respectively (1 to 2 CFU/reaction). To evaluate clinical applicability, 341 acid-fast bacilli in blood culture broths were analyzed. In total, 327 (96%) were positively identified: 54.5% *M. tuberculosis* complex, 37.5% *M. avium*, and 3.8% *M. intracellulare*. Results can be available within 3 hours of receiving a broth sample, which makes this rapid and simple assay an attractive diagnostic tool for clinical use. *(J Mol Diagn 2009, 11:42–48; DOI: 10.2353/jmoldx.2009.080081)***

*Mycobacterium tuberculosis* and *M. avium-intracellulare* complex are now the most common causes of systemic bacterial infection in AIDS patients. Automated broth culture rapidly recovers mycobacteria from infected blood. Molecular methods offer advantages over conventional methods such as improved accuracy in identification of *Mycobacterium* species and shorter turnaround time. Currently, molecular methods are widely used in many microbiological laboratories for pathogenic identification. There are commercial kits and in-house assays based on DNA hybridization probes for detecting mycobaterial DNA or RNA with or without nucleic acid amplification. Although these assays have been useful for identifying mycobacteria, most were applicable only for detecting one species per reaction and not recommended for use on blood-contaminated samples.[1–6]

The 16S rRNA gene is highly conserved and contains genus- and species-specific sequence variations useful for identifying different mycobacterial species. Among mycobacterial isolates from the blood of 385 AIDS patients at Siriraj Hospital and Bamrasnaradura Institute in 2001, 148 (38%) were *M. tuberculosis* and the rest were non-tuberculosis mycobacteria (NTM).[7] Additionally, we sequenced the 16S rRNA gene from NTM isolates, which revealed 180 (46%) *M. avium*, 39 (10%) *M. intracellulare*, 13 (3.3%) *M. simiae*, 11 (2.8%) *M. sherrisii*, 3 (0.8%) *M. kansasii*, and 1 (0.3%) *M. chelonae*/abscessus cases. Analysis of 39 *M. intracellulare* strains sequenced using hypervariable region A yielded 3 sequivars (distinct nucleic acid sequences), of which 18 (50%), 15 (38.5%), and 6 (15.5%) were identical to strain sequence database accession numbers AJ536036, AY652954, and AY652960, respectively. Both *M. tuberculosis* and *M. avium* did not contain sequivars in this region.

We designed a multihybridization probe real-time PCR assay targeting the 16S rRNA gene to facilitate identification of *M. tuberculosis* complex, *M. avium* and *M. intracellulare* in primary blood culture broth using the LightCycler PCR system (Roche Applied Science, Mannheim, Germany). This system acquired amplification fluorescence along with melting temperature (Tm) curves for the probe-PCR product complex. Tm analysis not only validates the fluorescence data but strengthens the assay especially in the case of *M. intracellulare* sequivars. Additionally, the assays have been validated on routine clinical blood culture broths.

## Materials and Methods

### Mycobacterial Strains and DNA Extraction

Mycobacterial strains included in this studies were either ATCC strains or clinical isolates (S) identified by 16S rRNA sequencing. These included *M. tuberculosis* (ATCC

Supported by Siiraj Grant Research and Development and Medical Education, Faculty of Medicine Siriraj Hospital, Mahidol University.

Accepted for publication October 14, 2008.

Address reprint requests to Suporn Foongladda, Mailing address: Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, 2 Prannok Rd., Bangkoknoi, Bangkok 10700, Thailand. E-mail: sisfm@mahidol.ac.th.

27294, H37Ra, H37Rv), *M. bovis* BCG Tokyo, *M. bovis* (ATCC 19210), *M. avium* (ATCC 700898), *M. intracellulare* (ATCC 13950 and 8 clinical isolates; S6, S9, S33, S34, S58, S77, S90, and S105), *M. kansasii* (ATCC 12478), *M. scrofulaceum* (ATCC 19981), *M. terrae* (ATCC 15750), *M. simiae* (ATCC 25275), *M. szulgai* (ATCC 35799), *M. xenopi* (ATCC19250), *M. sherrisii* (5 clinical isolates; S24, S53, S75, S81, and S155), *M. chelonae* (clinical isolate S12), and *M. abscessus* (clinical isolate S16). They were cultured on Löwenstein- Jensen (L-J) medium. Cell suspensions were prepared in PBS. DNA was extracted using High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to manufacturer's instructions. Extracted DNA was stored at −20°C.

## *Blood Culture Broth Samples and DNA Extraction*

A total of 341 individual routine acid-fast bacilli (AFB)-positive blood culture broths, which were either in 129 BACTEC Myco/F-lytic broths (Becton Dickinson, Sparks, MD) or 212 BacT/ALERT MB broths (bioMérieux, Durham, NC) were obtained from the Mycobacteria Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital and the Microbiology Laboratory, Bamrasnaradura Institute, Department of Disease Control, Ministry of Public Health, Thailand. Once automated machines confirmed growth, broth aliquots were used to inoculate a L-J solid medium and to prepare a smear for Ziehl-Neelsen staining. The remaining portion was used for DNA extraction. L-J mediums were incubated at 37°C and inspected weekly for growth. Conventional biochemical tests identified the acid-fast bacilli from L-J mediums for *M. tuberculosis*. PCR–restriction fragment-length polymorphism of the hsp65 gene detected individual NTM species. Lastly, unidentifiable bacilli were sequenced.[8,9]

DNA was extracted from 200 μl of a blood culture broth sample using the High Pure PCR Template Preparation Kit according to the manufacturer's instructions with modifications. Briefly, the sample was centrifuged and the pellet was suspended in 200 μl of PBS. Subsequently, lysis buffer with proteinase K was added. The sample underwent five freeze thaw cycles (boiled for 5 minutes, vortexed, and incubated for 2 minutes in a −70°C ethanol bath) followed with a final 5 minutes boiling step. DNA was eluted with 100 μl pre-warmed elution buffer. The rest of the steps were according to manufacturer's instructions. The elution was stored at −20°C.

## *Primers and Probes*

The primer and hybridization probe designs were based on alignments of the 16S rRNA genes of *Mycobacterium* spp. as shown in Figure 1, which included sequences from various mycobacterial species from the National Center for Biotechnology Information database (*http://www.ncbi.nlm.nih.gov*) and the clinical isolates: *M. tuberculosis* (BX842576), *M. avium* (FE059905), *M. intracellulare* 1 (AJ536036), *M. intracellulare* 2 (AY652954), *M. intracellulare* 3 (AY652960), *M. simiae* (X52931), *M. scrofulaceum* (AF480604), *M. sherisii* (AY353699), *M. kansasii* (X15916), *M. gastri* (AF080602), *M. genavens* (X60070), *M. hemophilum* (U06638), *M. marinum* (AY513243), *M. ulcerans* (AF547979), *M. microti* (AF480584), *M. szulgai* (X52926), *M. triplex* (U57632), *M. fortuitum* (AY457068), *M. chelonae* (AF547909), *M. abscessus* (DQ866776), *M. godonae* (EF428556), and *M. xenopi* (AF547982). The designations in parentheses are GenBank accession numbers.

The LightCycler Probe Design software 2.0 from Roche Applied Science was used to design optimal primers and probes for the system. The PCR assay included multiple species-specific probes to facilitate species identification by means of amplification curve detection and specific melting peaks. The sequences of the corresponding species and the sequences of the primers and probes are shown in Figure 1 and Table 1. A BLAST search (*http://www.ncbi.nlm.nih.gov/BLAST/*) was performed to check the specificity of primers and probes to the target DNA. The sequences of the forward and reverse primers recognized a ~320-bp region of the 16S rDNA. Sequence specific hybridization probes were designed Tb-fl/705-T and M-fl/640-A for *M. tuberculosis* complex and *M. avium*, respectively. Probe designated for *M. intracellulare*, 610-I, had A/G wobble at position 15 to perfectly match sequivars 1 and 3 and only one mismatch with sequivar 2 as shown in Figure 1 and Table 1. The upstream oligonucleotide probes (Tb-fl and M-fl) were labeled with fluorescein at the 3′ terminus. The downstream oligonucleotide probes (705-T, 640-A, and 610-I) were labeled with different shades of LightCycler Red at the 5′ terminus and phosphorylated at the 3′ terminus (TIBMOLBIOL, Berlin, Germany).

## *Real-Time PCR Assay*

The real-time PCR mixture contained 4 μl of commercial ready-to-use reaction mix for PCR (LightCycler FastStart DNA MasterPLUS Hybridization Probes kit; Roche Molecular Biochemicals, Mannheim, Germany), 0.5 μmol/L of each primer, 0.1 μmol/L of each probe and 2 μl of DNA extract with nuclease-free water added to a final volume of 20 μl. Each set of experiments included negative and positive controls. Nuclease-free water replaced DNA template for negative controls. Positive controls were prepared by adding 2 μl of template DNA from a *M. tuberculosis* H37Rv and *M. avium* ATCC 700898 mixture or *M. intracellulare* ATCC 13950. Reactions were performed on the LightCycler Real-Time PCR system, version 2.0. The cycling conditions included initial FastStart TaqDNA polymerase activation phase at 95°C for 10 minutes followed by a 40-step amplification procedure delineated into 10 seconds at 95°C, 20 seconds at 50°C, and 13 seconds at 72°C. Subsequently, melting analysis of the probe-PCR product duplex consisted of 95°C for 30 seconds followed by cooling to 40°C for 30 seconds before the temperature was raised to 75°C at a rate of 0.1°C/s with continuous fluorescence acquisition. The final cooling step was 40°C for 10 seconds. Downstream oligonucleotide probes were labeled with varying LightCycler Red dye and for that reason, amplified products were measured through different channel modes of emitted energy such as 640 nm, 705 nm, and 610

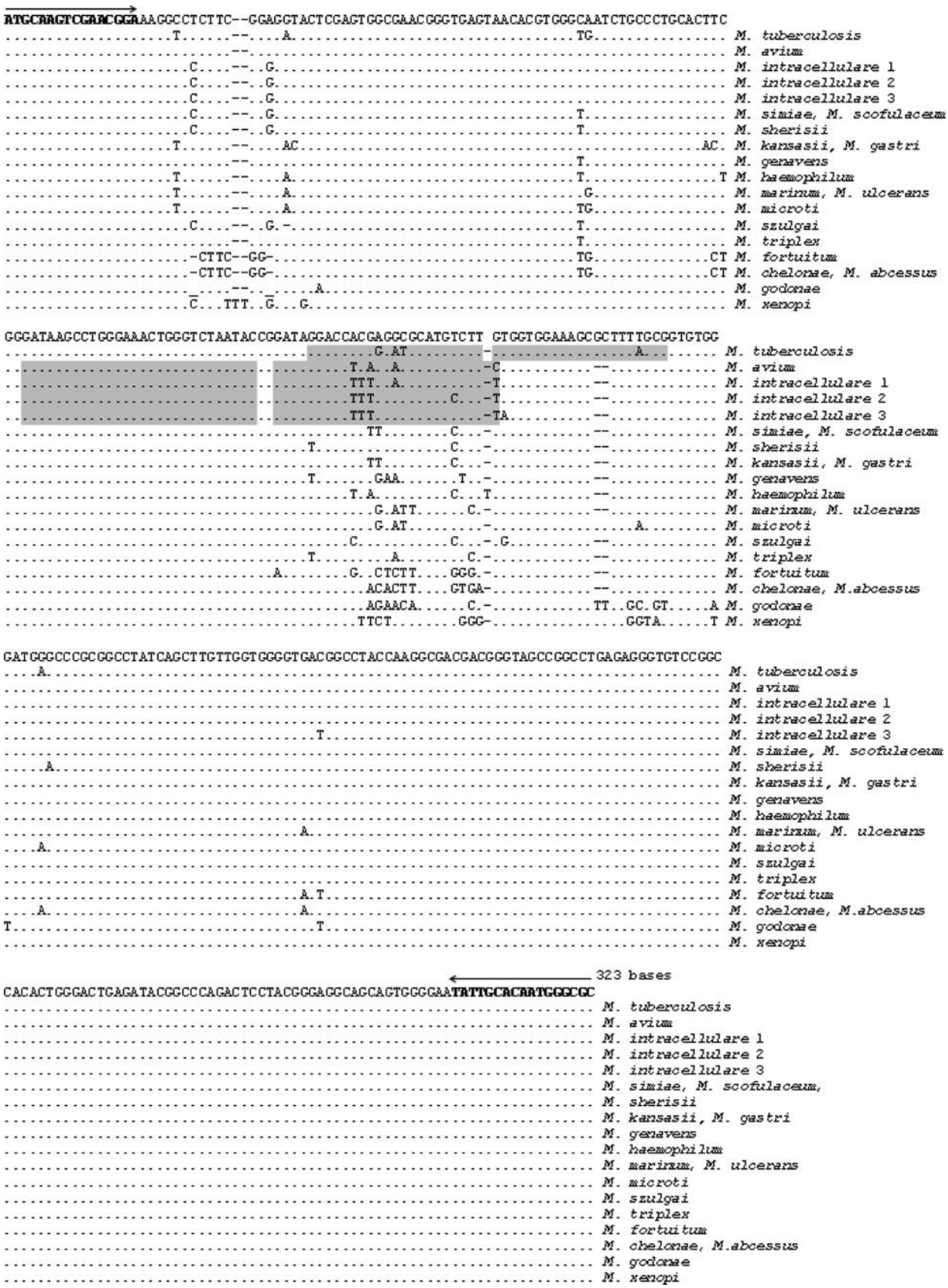


**Figure 1.** Alignment of 16S rRNA gene fragments of various mycobacterial species from the NCBI database and the clinical isolates. Nucleotides corresponding to the primers are in bolds. Sequences for the upstream and downstream hybridization probes are in shadows. The first consensus nucleotide corresponds to *E. coli* 16S rRNA gene (GenBank accession number M25051) position 55. Dot indicates identity and dash indicates deletion from the above consensus sequence.

**Table 1.** The Primers and Hybridization Probes Sequences for the Real-Time PCR Assays

| Name | Sequences | Species |
|---|---|---|
| Primers | | |
| Forward | 5′-ATGCAAGTCGAACGGA | *Mycobacterium* species |
| Reverse | 5′-GCGCCCATTGTGCAATA | *Mycobacterium* species |
| Upstream oligonucleotide probes | | |
| Tb-fl | 5′-GGACCACGGGATGCATGTCT-FL | *M. tuberculosis* complex |
| M-fl | 5′-GATAAGCCTGGGAAACTGGGTCTAATAC-FL | *M. avium*, *M. intracellulare* |
| Downstream oligonucleotide probes | | |
| 705-T | 5′-LC 705-GTGGTGGAAAGCGCTTTAGCG-PH | *M. tuberculosis* complex |
| 640-A | 5′-LC 640-GATAGGACCTCAAGACGCATGTCTTC-PH | *M. avium* |
| 610-I | 5′-LC 610-GATAGGACCTTTAGACGCATGTCTTT-PH (or G) | *M. intracellulare* |

LC, LightCycler Red; FL, fluorescein; PH, 3′ phosphate extension. For primer 610-1, wobble position 15 A or G.

nm. Tm was recorded and the presence of ~320-bp amplification products was verified by 2% agarose gel electrophoresis.

### *Determination of Sensitivity*

To determine detection limits of our assay, 2 ml blood culture negative broth or EDTA-blood of health volunteers were spiked with *M. tuberculosis* H37Rv, *M. avium* ATCC 700898, or *M. intracellulare* ATCC 13950 to a final concentration of $10^6$ CFU/ml. These samples were serially diluted tenfold down to $10^2$ CFU/ml and performed in duplicates. DNA was extracted using 200 $\mu$l of each sample.

### *Direct Identification of Mycobacteria in Positive Blood Culture Broth*

Initially, the positive blood culture broth was screened for the presence of *M. tuberculosis* or *M. avium*. *M. tuberculosis* complex probes (Tb-fl and 705-Tb) and *M. avium* probes (M-fl and 640-A) were added in one reaction mixture. Fluorescence signals from specific probes for *M. tuberculosis* complex and *M. avium* were detected in channels 705 and 640, respectively. Amplification curves in the 705 channel indicated positive for *M. tuberculosis* complex while amplification in the 640 channel denoted positive for the *M. avium* species. The Tm curves were simultaneously analyzed to confirm findings. In cases that were negative for these species, the samples were tested for presence of *M. intracellulare* using a second reaction tube containing M-fl and 610-I probes. Both amplification in channel 610 and Tm peaks within the range 57°C to 67°C must be observed for positive identification of *M. intracellulare*.

## *Results*

### *Specificity of Probes and Melting Temperature Values*

The BLAST searches revealed the *M. tuberculosis* probes, TB-fl and 705-T, were 100% sequence specific to all *M. tuberculosis* complex sequences in GenBank database, which includes 11 *M. tuberculosis*, 7 *M. bovis,* 3 *M. africanum*, 2 *M. microti*, 1 *M. canettii*, and 1 *M. pinnipedii*. The *M. avium* probes, M-fl and 640-T, were 100% specific to all 39 *M. avium* strains in the database. The blast search with the *M. intracellulare* probes, M-fl and 610-I, revealed 100% specificity to *M. intracellulare* as well as *M. chimaera.* This is a novel species in the *Mycobacterium avium* complex with 16S rRNA sequence similar to *M. intracellulare* 3 or ATCC 13950 as shown in Figure 1.[10]

The specificity of all primers and probes was tested on DNA from 12 mycobacterial species shown in Table 2. We confirmed ~320 bp PCR products on gel electrophoresis (data not shown). Fluorescence signals were detected in respective channel, which depended on the shade of LightCycler Red dye on the probe. Tm measurements were collected ranging from 40°C to 75°C. At temperatures lower than expected probe-PCR product complex Tm, melting peaks of non-specific products were observed in some cases. In positive samples, distinct secondary peaks occurred, which were more pronounced in samples with high concentrations of specific PCR products (Table 2 and Figure 2).

For assays using probes specific for the *M. tuberculosis* complex or *M. avium*, only the amplification products of *M. tuberculosis* complex or *M. avium* were detected in 705 or 640 channels, respectively. The target-specific Tms were 57.4°C /65.2°C (corresponds to primary/secondary peaks) for *M. tuberculosis* complex and 56°C /58.5°C for *M. avium* shown on Table 2. No cross-reactivity was observed with other mycobacterial species. In some cases where non-specific melting peaks were observed, the Tms were at least 8°C to 10°C lower as illustrated in Figure 2, A and B.

M-fl and 610-I probes detected all 3 *M. intracellulare* sequivars: 1) *M. intracellulare* 1 (S6, S33, S90) 2) *M. intracellulare* 2 (S9, S58, S77) 3) *M. intracellulare* 3 (ATCC 13950, S34, S105). The corresponding Tm values were 57°C /65.5°C , 57.5°C , and 60.7°C /67.3°C , respectively (Table 2). These probes showed cross-reactivity with *M. avium* at Tm value of 57.9°C. Positive signals were present in *M. simiae, M. kansasii, M. scrofulaceum, M. szulgi* and *M. xenopi* with non-specific melting peaks between 51°C to 54°C (Figure 2C).

### *Sensitivity*

Dilutions of mycobacterial culture in broth samples were prepared to determine the lowest mycobacterial load for

**Table 2.** *Mycobacterium* Species Tested by the Real-Time PCR Procedure

| *Mycobacterium* species | Amplification curve* and melting temperature (°C) detection with probes specific for | | | | | |
|---|---|---|---|---|---|---|
| | *M. tuberculosis* | | *M. avium* | | *M. intracellulare* | |
| *M. tuberculosis* complex[a] | + | 57.4/65.2 | − | <40 | − | 48.7 |
| *M. avium* | − | 44 | + | 56/58.5 | + | 57.9 |
| *M. intracellulare* 1[b] | − | 43 | − | 40.2 | + | 57/65.5 |
| *M. intracellulare* 2[c] | − | 45.8 | − | 50.1 | + | 57.5 |
| *M. intracellulare* 3[d] | − | 42.5 | − | 47.2 | + | 60.7/67.3 |
| *M. simiae* | − | 45.6 | − | <40 | + | 53.9 |
| *M. kansasii* | − | 46.4 | − | <40 | + | 53.8 |
| *M. sherrisii* | − | 46.4 | − | <40 | − | 50.1 |
| *M. terrae* | − | 51.1 | − | <40 | − | <40 |
| *M. scrofulaceum* | − | 46.5 | − | <40 | + | 54.1 |
| *M. szulgai* | − | 43.9 | − | <40 | + | 52.9 |
| *M. xenopi* | − | <40 | − | <40 | + | 51.8 |
| *M. chelonae* | − | <40 | − | <40 | − | <40 |
| *M. abscessus* | − | <40 | − | <40 | − | <40 |

*Amplification curve was detectable (+), not detectable (−), or weak signal (±).
[a]*M. tuberculosis* ATCC 27294, *M. bovis* BCG Tokyo, and *M. bovis* ATCC 19210.
[b]*M. intracellulare* 1, S6, S33, S90: sequence at sensor probe position is 5′-GATAGGACCTTTAGACGCATGTCTTT-3′.
[c]*M. intracellulare* 2, S9, S58, S77: sequence at sensor probe position is 5′-GATAGGACCTTTAGGCGCATGCCTTT-3′.
[d]*M. intracellulare* 3, ATCC 13950, S34, S105: sequence at sensor probe position is 5′-GATAGGACCTTTAGGCGCATGTCTTT-3′.

a positive signal. Detection limits for *M. tuberculosis*, *M. avium*, and *M. intracellulare* were 350, 600 and 650 CFU/ml (0.7, 1.2 and 1.3 CFU per reaction), respectively. The assay sensitivity in mycobactria spiked blood samples for *M. tuberculosis* and *M. avium* was 800 and 2000 CFU/ml (1.6 and 4 CFU per reaction), respectively. Analytical sensitivity was not compromised when the *M. tuberculosis* and *M. avium* probes were combined in one reaction mixture.

## *Clinical Application in Positive Blood Culture Broth*

Multiprobe real-time PCR assay was used to identify 341 positive clinical specimens from the automated blood broth system. Results from biochemical and PCR assays (amplification and Tm values) are presented in Table 3 and Figure 3. Initial reaction correctly identified *M. tuberculosis* complex ($n$ = 186, 54.5%) and *M. avium* ($n$ = 128, 37.5%) positive samples, which matched corresponding results from conventional identification assays. The negative samples were further investigated for *M. intracellulare* in a second reaction. Thirteen samples were positive for *M. intracellulare* where 8 samples with corresponding Tm values of *M. intracellare* sequivar 1, 3 of *M. intracellare* sequivar 2, and 2 of *M. intracellare* sequivar 3 were observed. One sample showed weak positive for *M. intracellulare* with Tm peak at 57.7°C but it's Tms with *M. tuberculosis* complex and *M. avium* probes were not in ranges of *M. intracellulare*. This sample was identified by sequencing as *M. hemophilum.* NTM isolates that were unidentifiable demonstrated lower Tms than expected Tms. These samples were identified by sequencing as *M. kansasii* ($n$ = 5, 1.5%), *M. sherrisii* ($n$ = 4, 1.2%), *M. simiae* ($n$ = 3, 0.9%), and *M. triplex* ($n$ = 1, 0.5%).

However, the 610-I probe designed for *M. intracellulare* sequivar 3 or ATCC 13950 had cross similarity with the recently published species, *M. chimaera*.[9] We performed hypervariable 16S rRNA gene sequencing on clinical isolates S34 and S105, which were positive for *M. intracellare* 3 to establish definitive identifications. Sequences were found to be identical to *M. intracellulare* (GenBank accession number AJ356036), which consisted of C at position 459 in hypervariable B region and not *M. chimaera* (T at position 459).

## *Discussion*

Rapid identification of disseminated mycobacterial infections in AIDS patients is important for early detection and treatment to improve patient outcome. A development of a high-throughput, sensitive diagnostic test performed on primary blood broth is crucial. Therefore, we sought to develop rapid molecular diagnostic assay, which is simple and specific for the detection of common pathogenic mycobacteria in primary blood culture broth.

Traditional identification methods are time-consuming from slow growing subculture colonies on solid media to using biochemical tests. Recently, molecular techniques such as nucleic acid probes, species-specific PCR, reverse hybridization, and 16S rRNA sequencing have been introduced that offer rapid and highly sensitive assays.[1,2,5,6,11–13] Many of these assays require up-front sample processing and are not tested directly on blood or blood culture broths. Commercial kits are also available for species identification but some kits are expensive and presence of blood in samples may interfere or compromise efficacy.[14–16] One such kit is the commercial acridinium-ester-labeled probe assay, which is easy to use and highly specific for the *M. tuberculosis* complex, but the probe has a high detection limit (approximately $10^6$ CFU/ml) and requires a separate reaction tube for *M. avium-intracellulare* complex identification.[17] Although multispecies identification is possible with the multiprobe reverse hybridization commercial assay, the procedure is laborious with high risk for PCR amplicon contamination, which is not ideal for a clinical laboratory.

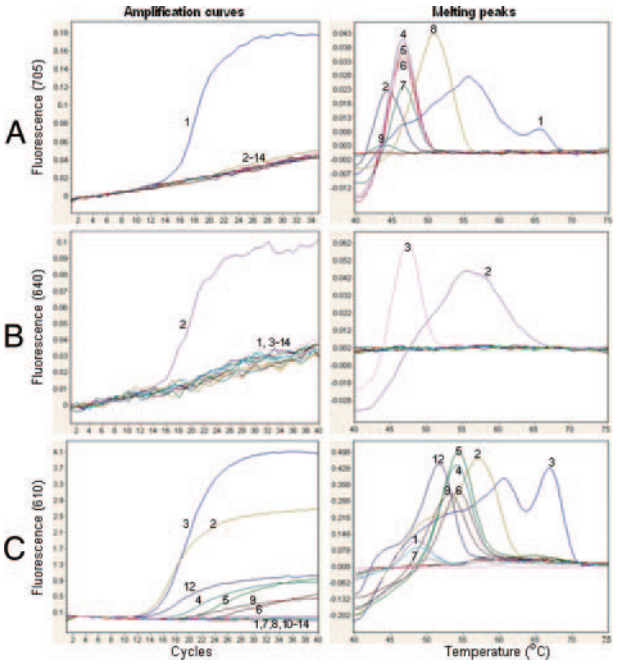


**Figure 2.** Real-time PCR amplification curves and the melting curves at channel 705 (**A**), 640 (**B**), and 610 (**C**) detections: 1. *M. tuberculosis* ATCC 27294; 2. *M. avium* ATCC 700898; 3. *M. intracellulare* ATCC 13950; 4. *M. kansasii* ATCC 12478; 5. *M. scrofulaceum* ATCC19981; 6. *M. simiae* ATCC 25275; 7. *M. sherrisii* S24; 8. *M. terrae* ATCC 15750; 9. *M. szulgai* ATCC 35799; 10. *M. chelonae* S12; 11. *M. abscessus* S16; 12. *M. xenopi* ATCC 19250; 13. *M. gordonae* ATCC 14470; and 14. negative control.

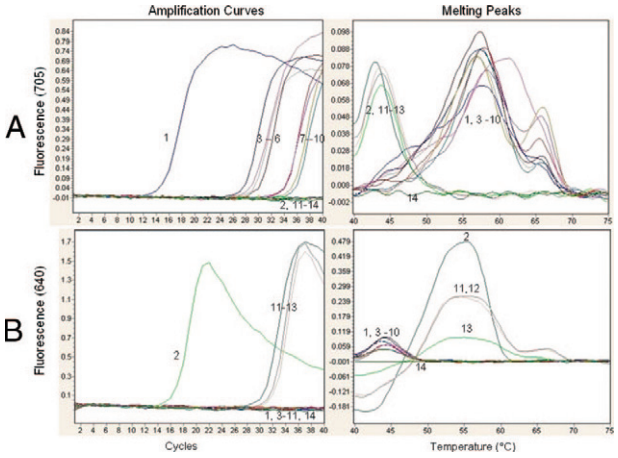


**Figure 3.** Real-time PCR amplification curves and melting curves channel 705-*M. tuberculosis* (**A**) and channel 640- *M. avium* (**B**) in the same reaction tube. 1. *M. tuberculosis* positive control; 2. *M. avium* positive control; 3. through 13., samples; and 14. negative control. Sample numbers 3 to 10 were identified as *M. tuberculosis* complex. Sample numbers 11 to 13 were identified as *M. avium*.

The LightCycler system has been developed for rapid identification of *M. tuberculosis* complex and *M. avium* but not for *M. intracellulare*.[1] However, the assay used one reaction per species and did not apply to use on blood containing samples. This system has also been developed to differentiate *M. tuberculosis* and NTM by using probe-PCR product Tm ranges in a single probe set.[2] Nevertheless, the assay could not identify the species of NTM because of the wide variety of NTM with Tm overlapping. The system has also been developed for rapid detection of rifampin and isoniazid resistance in *M. tuberculosis*.[3]

We designed the PCR assay on a LightCycler for several reasons. It is one of the fastest real-time PCR systems. Additionally, the ability to collect data through multiple channels allows for discrimination of various probe-amplicon complexes and reports their Tms. Two sets of hybridization probes were included in a single reaction tube to provide an efficient, simultaneous differentiation of *M. tuberculosis* complex and *M. avium* in one LightCycler run. When the samples were negative for *M. tuberculosis* complex and *M. avium*, they were subsequently tested for *M. intracellulare*. Tm analysis was essential in identifying not only the mycobacterial species but to differentiate the three sequivars of *M. intracellulare*.

Modifications were made to the DNA extraction protocol with the High Pure PCR Template Preparation Kit. We determined that following this modified process is imperative in successfully detecting mycobacterial DNA from samples with blood. The most significant change was the use of mechanical force to lyse the cells via the freeze-thaw cycles. In this study, detection limits were 1 to 2 CFU per test, and 2 to 4 CFU per test, for blood culture broth and whole blood, respectively. This highly sensitive assay is a direct result of the high quality DNA template. Bacterial concentration in the blood circulation is inconsistent, thus the exact blood volume necessary for direct testing on blood is difficult to ascertain.

**Table 3.** Mycobacterial Samples in the Primary Blood Culture Broth Identified by Real-Time PCR Compared with the Results of Culture Analysis

| | | Real-Time PCR | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Culture results | No of samples | Ac | Channel 705 Tm (°C ) | Ac | Channel 640 Tm (°C ) | Ac | Channel 610 Tm (°C ) | Interpretation |
| *M. tuberculosis* | 186 | 186 | 59 ± 1.3/65 ± 0.6 | 0 | 44.7 ± 0.7 | nd | nd | *M. tuberculosis* complex |
| *M. avium* | 128 | 0 | 44 ± 0.6 | 128 | 56 ± 1.4/60.4 ± 2 | nd | nd | *M. avium* |
| *M. intracellulare* | 13 | 0 | 42–43 | 0 | 43–51 | 13 | 58 ± 0.7/66 ± 0.6 | *M. intracellulare* |
| *M. kansasii* | 5 | 0 | 46 | 0 | <40 | 0 | 53–54 | Non TB/MAC |
| *M. sherrisii* | 4 | 0 | 45–46 | 0 | <40 | 0 | <40 | Non TB/MAC |
| *M. simiae* | 3 | 0 | 41–43 | 0 | <40 | 0 | 53–54 | Non TB/MAC |
| *M. haemophilum* | 1 | 0 | 48.2 | 0 | 53.7 | 1 | 57.7 | Non TB/MAC |
| *M. triplex* | 1 | 0 | 51.3 | 0 | 50.9 | 0 | <40 | Non TB/MAC |

Ac, Number of samples showed positive amplification curves; Tm, Melting temperature; nd, not done; Non TB/MAC, Neither *M. tuberculosis* complex nor *M. avium* complex.

Our multiprobe real-time PCR assay was successful in identifying samples from primary blood broths without reduction in sensitivity. The probes specific for *M. tuberculosis* complex and *M. avium* showed 100% accuracy in the first reaction. Although *M. intracellulare* probe in the second reaction could not differentiate between *M. intracellulare* 2 sequence and *M. avium* (Tms in 610 was ~57°C), since *M. avium* is confirmed in the first run positive samples were not included in the second reaction pool. Even if Tm of *M. hemophilum* was in the same range of *M. intracellulare*, we could discriminate by its Tms in 705 and 640 that were higher than *M. intracellulare*. In total, 96% of the samples were correctly identified making this simple PCR assay a robust, highly specific diagnostic tool in differentiating commonly isolated mycobacteria from blood cultures of AIDS patients.

In addition, our method provides a cost-effective and efficient alternative to conventional techniques and commercial kits currently in use. The ability to work on primary blood culture broth and blood decreases the up-front sample preparation time. On receiving a patient sample, it takes approximately 3 hours (DNA extraction 1 hour, reaction preparation 20 minutes, and LightCycler 50 minutes) to identify these three common species of mycobacteria by this semiautomated system. The ability to modify this assay such as designing new detection probes for other species is paramount to expand its use to account for endemic diseases and new outbreaks in various clinical laboratories.

This assay will be evaluated to detect mycobacterial pathogen directly on patient specimens such as blood, sputum, cerebrospinal fluid and stool. In order to identify active disease, targeting RNA that degrades rapidly compared to DNA will help to evaluate current disease activity in a patient even after drug therapy.

Within the limitations of this study, it may be concluded that this multiprobe real-time PCR assay is an accurate, rapid and simple method for identifying the most common mycobacteria species directly in positive blood culture broths. This assay is useful for the diagnosis of *M. tuberculosis* and *M. avium-intracellulare* complex from AIDS disseminated mycobacteriosis but alternate molecular techniques such as DNA sequencing should be used to identify other infrequent species.

## *Acknowledgments*

We thank Dr. Mami Taniuchi, Professor Dr. John J Boitano and Associate Professor Dr. Eric Houpt for their advice and English editing.

## *References*

1. Lachnik J, Ackermann B, Bohrssen A, Maass S, Diephaus C, Puncken A, Stermann M, Bange FC: Rapid-cycle PCR and fluorimetry for detection of mycobacteria. J Clin Microbiol 2002, 40:3364–3373
2. Shrestha NK, Tuohy MJ, Hall GS, Reischl U, Gordon SM, Procop GW: Detection and differentiation of Mycobacterium tuberculosis and non-tuberculous mycobacterial isolates by real-time PCR. J Clin Microbiol 2003, 41:5121–5126
3. Torres MJ, Criado A, Palomares JC, Aznar J: Use of real-time PCR and fluorimetry for rapid detection of rifampin and isoniazid resistance-associated mutations in mycobacterium tuberculosis. J Clin Microbiol 2002, 38:3194–3199
4. Tortoli E, Bartoloni A, Böttger EC, Emler S, Garzelli C, Magliano E, Mantella A, Rastogi N, Rindi L, Scarparo C, Urbano P: Burden of unidentifiable mycobacteria in a reference laboratory. J Clin Microbiol 2001, 39:4058–4065
5. Cleary TJ, Roudel G, Casillas O, Miller N: Rapid and specific detection of mycobacterium tuberculosis by using the smart cycler instrument and a specific fluorogenic probe. J Clin Microbiol 2003, 41:4783–4786
6. Miller N, Cleary T, Kraus G, Young AK, Spruill G, Hnatyszyn HJ: Rapid and specific detection of mycobacterium tuberculosis from acid fast bacillus smear-positive respiratory specimens and BacT/ALERT MP culture bottles by using fluorogenic probes and real-time PCR. J Clin Microbiol 2002, 40:4143–4147
7. Foongladda S, Prasong N, Eampokalap B, Sutthent R: 16S rRNA gene sequence species and drugs susceptibility of disseminated non-tuberculous mycobacterial infection in AIDS patients. Int J Infect Dis 2004, 8 Suppl 1:S53
8. Vencent V, Drown-Elliot BA, Jost KC, Wallace RJ: Mycobacterium: phenotypic and genotypic identification. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH, eds. Manual of clinical microbiology, 8th edn. Washington DC: ASM Press, 1999:399–437
9. Telenti A, Marchesi F, Balz M, Bally F, Bfttger EC, Bodmer T: Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol 1993, 31:175–178
10. Tortoli E, Rindi L, Garcia MJ, Chiaradonna P, Dei R, Garzelli C, Kroppenstedt RM, Lari N, Mattei R, Mariottini A, Mazzarelli G, Murcia MI, Nanetti A, Piccoli P, Scarparo C: Proposal to elevate the genetic variant MAC-A, included in the Mycobacterium avium complex, to species rank as Mycobacterium chimaera sp. nov. Int J Syst Evol Microbiol 2004, 54:1277–1285
11. Kirschner P, Rosenau J, Springer B, Teschner K, Feldmann K, Böttger EC: Diagnosis of mycobacterial infections by nucleic acid amplification: 18-month prospective study. J Clin Microbiol 1996, 34:304–312
12. Miller N, Cleary T, Kraus G, Young AK, Spruill G, Hnatyszyn HJ: Rapid and specific detection of Mycobacterium tuberculosis from acid fast bacillus smear-positive respiratory specimens and BacT/ALERT MP culture bottles by using fluorogenic probes and real-time PCR. J Clin Microbiol 2002, 40:4143–4147
13. Taylor MJ, Hughes MS, Skuce RA, Neill SD: Detection of Mycobacterium bovis in bovine clinical specimens using real-time fluorescence resonance energy transfer probe rapid-cycle PCR. J Clin Microbiol 2001, 39:1272–1278
14. Scarparo C, Piccoli P, Rigon A, Ruggiero G, Nista D, Piersimoni C: Direct identification of mycobacteria from MB/BacT alert 3D bottles: comparative evaluation of two commercial probe assays. J Clin Microbiol 2001, 39:3222–3227
15. Ephraim DA, Spitzer ED: Use of acridinium-ester-labeled DNA probes for identification of mycobacteria in Bactec13A blood cultures. Diagn Microbiol Infect Dis 1994, 18:137–139
16. Evans KD, Nakasone AS, Sutherland PA, de la Maza LM, Peterson EM: Identification of Mycobacterium tuberculosis and Mycobacterium avium-M. intracellulare directly from primary BACTEC cultures by using acridinium-ester-labeled DNA probes. J Clin Microbiol 1992, 30:2427–2431
17. Smith MB, Bergmann JS, Woods GL: Detection of Mycobacterium tuberculosis in BACTEC 12B broth cultures by the Roche Amplicor PCR assay. J Clin Microbiol 1997, 35:900–902