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Detection and discrimination of *Mycobacterium tuberculosis* complex☆

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Abstract

A real-time quantitative polymerase chain reaction (qPCR) was developed for detection and discrimination of *Mycobacterium tuberculosis* (H37Rv and H37Ra) and *M. bovis* bacillus Calmette–Guérin (BCG) of the *Mycobacterium tuberculosis* complex (MTBC) from mycobacterial other than tuberculosis (MOTT). It was based on the melting curve (Tm) analysis of the *gyrB* gene using SYBR® Green I detection dye and the LightCycler 1.5 system. The optimal conditions for the assay were 0.25 μmol/L of primers with 3.1 mmol/L of $MgCl_2$ and 45 cycles of amplification. For *M. tuberculosis* (H37Rv and H37Ra) and *M. bovis* BCG of the MTBC, we detected the crossing points (Cp) at cycles of 16.96 ± 0.07, 18.02 ± 0.14, and 18.62 ± 0.09, respectively, while the Tm values were 90.19 ± 0.06 °C, 90.27 ± 0.09 °C, and 89.81 ± 0.04 °C, respectively. The assay was sensitive and rapid with a detection limit of 10 pg of the DNA template within 35 min. In this study, the Tm analysis of the qPCR assay was applied for the detection and discrimination of MTBC from MOTT.

Keywords: *Mycobacterium tuberculosis* complex (MTBC); *gyrB* gene; SYBR® Green I; LightCycler

1. Introduction

Tuberculosis (TB) is a disease that causes the deaths of approximately 2 million people per year worldwide (Raviglione, 2003). TB in humans is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) and other closely related species and subspecies including *M. bovis*, which is the causative agent of bovine tuberculosis; *M. bovis* bacillus Calmette–Guérin (BCG), which is the live, attenuated tuberculosis vaccine strain; and *M. africanum*, which is commonly isolated in African countries. These strains are collectively known as the *M. tuberculosis* complex (MTBC) and are the causative agents of TB in humans. MTBC is composed of mycobacteria with genetically identical 16S rRNA (Böddinghaus et al., 1990) and greater than 99.9% identical nucleotides (Sreevatsan et al., 1997), unlike other mycobacterial species (Ernst et al., 2007).

Since the 1980s, many other mycobacterial, known as nontuberculous mycobacterial (NTM) or mycobacterial other than tuberculosis (MOTT), have also been associated with human disease with increasing frequency worldwide (Wang and Tay, 1999). The *M. avium–M. intracellulare* complex (MAC) of MOTT causes opportunistic infection in immunocompromised patients who are infected with the HIV virus (Ratlegde et al., 1985). Therefore, differentiation between MOTT and MTBC is required to diagnose mycobacterial infection in AIDS patients.

Molecular methods for nucleic acid amplification such as polymerase chain reaction (PCR) or real-time quantitative PCR (qPCR) assays have been widely used to identify organisms with rapid and sensitive detection with small sample volume (Slinger et al., 2007). The qPCR method is superior to the PCR method because qPCR chemistries allow for the detection and measurement of the reaction kinetics during the early phases of PCR amplification (Tell et al., 2003). Application via the qPCR method in mycobacterial DNA studies includes the detection, species

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differentiation, and quantification of the mycobacterial DNA from the clinical samples (Drosten et al., 2003; Fang et al., 2002; van Coppenraet et al., 2004). The use of hydrolysis probe qPCR and PCR assays for the detection of tuberculosis in our laboratory has been previously published (Suhaila et al., 2008).

The *gyrB* gene encoding the subunit B protein of DNA gyrase (topoisomerase type II) is a suitable phylogenetic marker for the identification and classification of the *Acinetobacter* genus (Yamamoto et al., 1999). With the use of the same concept for the detection of MTBC, the *gyrB* gene containing 4 synonymous substitutions can be used as a phylogenetic marker (Niemann et al., 2000). The *gyrB* gene has been applied in the PCR using universal primer and PCR-restriction fragment length polymorphism analysis to differentiate MTBC from other slow-growing mycobacterial species (Kasai et al., 2000).

Previously, culture and smear methods have been extensively used to detect mycobacterial species from clinical specimens. However, the culture method to detect and differentiate among the MTBC species is time consuming. In this study, we applied the qPCR that was based on the melting curve (Tm) analysis of the *gyrB* gene to detect and discriminate MTBC from MOTT.

2. Materials and methods

2.1. Bacterial extraction and primers for qPCR

The mycobacterial species were from the American Type Culture Collection (ATCC) or IMR Culture Collection at the Institute for Medical Research (IMR) in Kuala Lumpur, Malaysia. These were cultured on Lowenstein–Jensen and Ogawa media and incubated at 37 °C. The DNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche, Germany) according to the manufacturer's instructions. Briefly, 200 μL of bacterial suspension was lysed, incubated at 72 °C for 15 min, and centrifuged at 14,000 rpm for 1 min. Inhibitor removal buffer was added, and the samples were centrifuged, washed, and eluted using 50 μL of the elution buffer. The eluted DNA was quantified using BioPhotometer (Eppendorf, Germany) according to the manufacturer's instructions.

2.2. Optimization assay

The primers targeting the 280-bp fragment of the *gyrB* gene that is specific for the MTBC were designed using Vector NTi® software (Invitrogen, USA). The primer sequences were MTBCF3_N (5′-AGG ACC GCA AGC TAC TGA AGG ACA-3′) and MTBCB3_N (5′-AAC TCT CGT GCC TTA CGT GCC C-3′). Conventional PCR was performed to assess the performance of the MTBCF3_N and MTBCB3_N primer pair during amplification. We combined 12.5 μL of 2X Superhot Mastermix (Bioron, Germany), 0.4 μL of 100 μmol/L $MgCl_2$, and 1.0 μL of 10 μmol/L primers with 2 μL of the DNA template in 25-μL reaction volumes. PCR assay was performed according to the following conditions: 95 °C for 10 min, followed by 35 cycles of 95 °C for 10 s, 66 °C for 15 s, and 72 °C for 15 s.

qPCR assay was performed using LightCycler® 1.5 (Roche). Optimization assay was performed using different concentrations of the primer set (0.25, 0.13, and 0.06 μmol/L) in the presence of 3.1 mmol/L of $MgCl_2$. The 20-μL reaction mixture contained 10X LightCycler® DNA Master SYBR® Green I (Roche) and 2 μL of the DNA template. Amplification was performed using the following conditions: a preincubation step at 95 °C for 30 s, 45 cycles of annealing at 66 °C for 10 s, and elongation at 72 °C for 15 s followed by Tm analysis with increasing temperatures from 65 °C to 95 °C in a 0.1 °C/s slope increment for 15 s. Different concentrations of *M. tuberculosis* H37Ra DNA (100 ng and 1 pg) were tested. Distilled water was used to replace the DNA template for the nontemplate control (NTC), and each set of assays was performed in triplicate samples (n = 3).

2.3. Sensitivity and specificity assays

The sensitivity assay was performed using a 10-fold dilution of *M. tuberculosis* H37Ra DNA (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, and 1 pg). The specificity assay was determined using 100 ng of the DNA template for the detection and discrimination of MTBC from MOTT. In this assay, *M. tuberculosis* H37Rv (ATCC 27294) and H37Ra (ATCC 25177), and *M. bovis* BCG (Institute Pasteur) were used as positive controls for MTBC. Other mycobacteria species for MOTT were *M. xenopi* (ATCC 19250), *M. smegmatis* (ATCC 14468), *M. kansasii* (ATCC 12478), *M. gordonae* (ATCC 14470), *M. avium* (M170/06), *M. flavescens* (ATCC 14474), *M. triviale* (T-254-3), *M. intracellulare* (ATCC 15984), *M. fortuitum* (M123/97), and *M. terrae* (W 45). *Bacillus subtilis* (ATCC 6633) and *Salmonella* species (S11/99) were used as for the nonmycobacterial species. Distilled water was used to replace the DNA template for the

Table 1
Effects of different concentrations of primers and DNA template on Cp and Tm values

Primer concentration (μmol/L)	DNA template (*M. tuberculosis* H37Ra)	Cp (cycle)[a]	Tm (°C)[b]
0.25	100 ng	17.25 ± 0.26	90.57 ± 0.06
	1 pg	34.09 ± 0.59	90.92 ± 0.05
	NTC	35.60 ± 0.29	82.73 ± 0.23
0.13	100 ng	20.18 ± 0.04	90.56 ± 0.08
	1 pg	≥40.00	90.48 ± 0.23
	NTC	ND	ND
0.06	100 ng	27.55 ± 0.61	90.30 ± 0.11
	1 pg	ND	ND
	NTC	ND	ND

[a]Cp and [b]Tm values are based on the averages of triplicate samples (n = 3).
ND = Not determined.

Table 2
Effects of different concentrations of the DNA template for the qPCR assay

DNA template		Cp (cycle)[a]	Tm (°C)[b]
NTC		35.51 ± 0.66	82.25 ± 0.17
M. tuberculosis H37Ra	100 ng	17.91 ± 0.10	90.61 ± 0.08
	10 ng	21.57 ± 0.40	90.72 ± 0.11
	1 ng	24.98 ± 0.17	90.45 ± 0.10
	100 pg	28.48 ± 0.16	90.17 ± 0.16
	10 pg	31.83 ± 0.06	90.06 ± 0.04
	1 pg	34.66 ± 0.70	90.09 ± 0.09
	100 fg	35.02 ± 0.14	90.20 ± 0.04

[a]Cp and [b]Tm values are based on the averages of triplicate samples (n = 3).

NTC, and each set of assays was performed in triplicate samples (n = 3).

2.4. Nucleotide sequencing and analysis

Amplified products of *M. tuberculosis* (H37Rv and H37Ra) and *M. bovis* BCG were verified using the Sanger sequencing method with the MTBCF3_N and MTBCB3_N primer set. For sequencing, the PCR products were purified using a PCR purification kit (Novagen), according to the manufacturer's instructions. The sequencing data were analyzed against the global database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the fragment based on species. The sequence was further analyzed using a multiple-sequence alignment with *M. tuberculosis* (H37Rv and H37Ra) and *M. bovis* BCG which were used as reference strains. The 220-bp fragments of the *gyrB* gene for *M. tuberculosis* (H37Rv and H37Ra) and *M. bovis* BCG were submitted and accepted (GenBank accession numbers of HM802261, HM802262, and HM802263, respectively).

3. Results and discussion

3.1. Optimization assay

Before proceeding with the optimization assay of qPCR, the annealing temperature was determined using conventional PCR (data not shown). The optimization assay of qPCR was performed for 45 cycles of amplification at an annealing temperature of 66 °C using the MTBCF3_N and MTBCB3_N primer set with 3.1 mmol/L of $MgCl_2$. The results show that the primer concentration in the reaction mixture affects the Tm analysis of the assay (Table 1). Based on the Tm analysis, the primer concentration was optimal at 0.25 μmol/L because the crossing point (Cp) value of 17.25 ± 0.26 was the lowest cycle observed. Therefore, this primer concentration (0.25 μmol/L) was used as the optimal primer condition for the subsequent qPCR assays.

The Cp values obtained for NTC and 1 pg of the DNA template were similar, indicating that the amplification of low concentrations of the DNA template was indistinguishable from NTC (Table 1) due to the formation of primer dimers. The Cp value was observed at 17.25 ± 0.26 cycles in the assay containing 100 ng of the DNA template, whereas the Cp value was 34.09 ± 0.59 cycles in the assay containing 1 pg of the DNA template. The Cp value observed for 1 pg of the DNA template was the cycle number that indicated the cycle when the primer dimers formed (Wittwer et al., 1997).

At 100 ng of the DNA template and different concentrations of the primer set, the Tm values were constant at 90.57 ± 0.06 °C, 90.56 ± 0.08 °C, and 90.30 ± 0.11 °C (Table 1). The observed Tm values were the result of specific reaction products because the reaction melted at

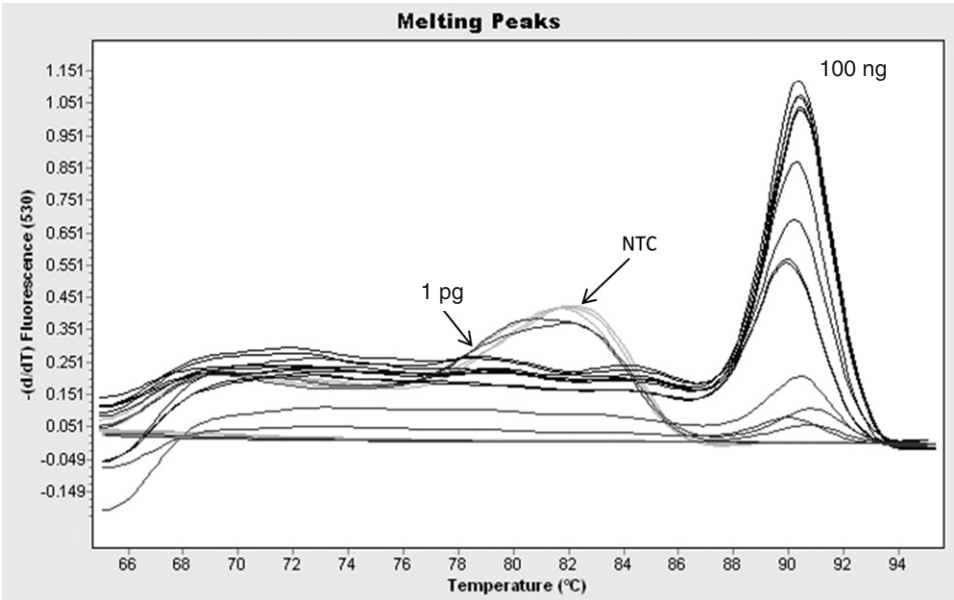

Fig. 1. Melting curve (Tm) analysis observed during the optimization of the qPCR assay (n = 3).

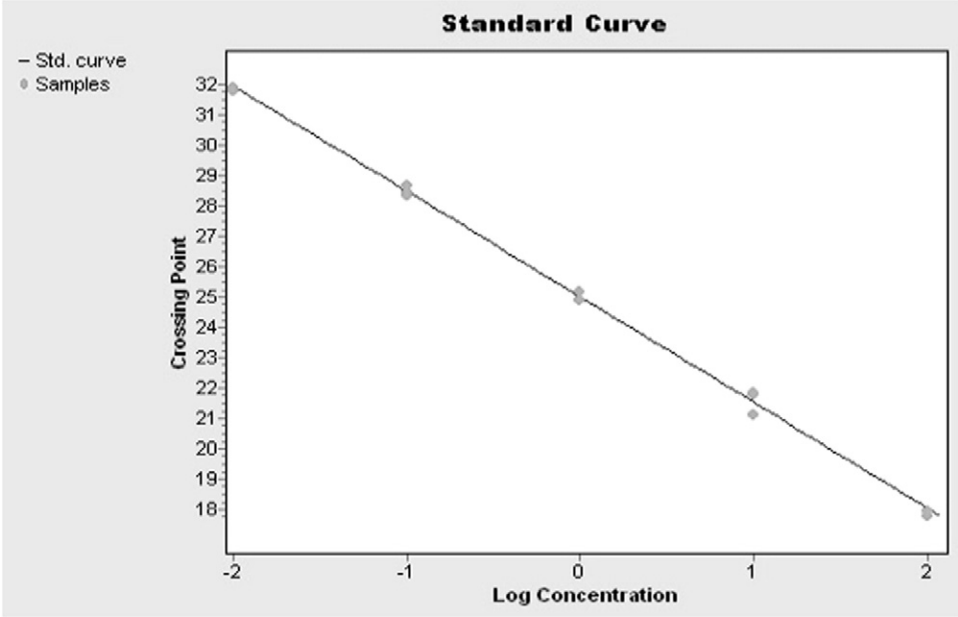

Fig. 2. Standard curve observed to assess the sensitivity of the qPCR assay (n = 3). Efficiency = 1.939; standard error = 0.021; and R^2 = 0.998.

higher temperature than the melting temperature of the primer dimers. The reaction product represented the specific amplified product of the *gyrB* gene (Niemann et al., 2000) with known high GC content of mycobacterial species as previously reported by Xu et al. (2000).

3.2. Sensitivity and specificity assays

The sensitivity assay of the qPCR was determined using a 10-fold dilution starting from 100 ng to 100 fg of the *M. tuberculosis* H37Ra DNA. The limit of detection was 10 pg of the DNA template (Table 2). These results are similar with the results obtained for the duplex PCR assay based on the *rpoB* gene (Kim et al., 2004). Primer dimers without any significant DNA product were detected during amplification at a low dilution of 1 pg of the *M. tuberculosis* H37Ra DNA (Fig. 1).

The Cp value for 10 pg of the DNA template was at 31.03 ± 0.05 cycles, whereas the Cp value for 100 ng of the DNA template was detected at a faster cycle of 17.92 ± 0.08. We concluded that the Cp value at above 35 cycles was accepted as a negative or inconclusive finding due to the greater variability and unreliable quantification. The 10-fold difference in the concentration corresponded to 3.42 ± 0.28 cycles, which was based on the average of each serial dilution. At different concentrations of the DNA template, the Tm value was constant at 90.61 ± 0.08 °C, 90.72 ± 0.11 °C, 90.45 ± 0.10 °C, 90.17 ± 0.16 °C, and 90.06 ± 0.04 °C for 100 ng, 10 ng, 1 ng, 100 pg, and 10 pg, respectively, in concordance with the results of the aforementioned optimization assay.

As shown in Fig. 2, the standard curve was generated using a serial dilution of the DNA template; the efficiency was 1.939 ± 0.021, standard error was 0.021, and reproducibility of the replicates (R^2) was 0.998, respectively. The intercept on the Cp axis indicated that 10 pg of the DNA template would be detected at a Cp value of 32 cycles. The amplification efficiency of the assay showed reliable quantification of the target gene in concordance with the sigmoid function of the product yield (Alvarez et al., 2007).

3.3. Detection and discrimination of MTBC

The Cp values had cycles of 16.96 ± 0.07, 18.02 ± 0.14, and 18.62 ± 0.09 for *M. tuberculosis* (H37Rv and H37Ra) and *M. bovis* BCG, respectively (Table 3, Fig. 3). We

Table 3
Detection and discrimination of MTBC

		Cp (cycle)[a]	Tm (°C)[b]
M. tuberculosis complex (MTBC)	*M. tuberculosis* H37Rv	16.96 ± 0.07	90.19 ± 0.06
	M. tuberculosis H37Ra	18.02 ± 0.14	90.27 ± 0.09
	M. bovis BCG	18.62 ± 0.09	89.81 ± 0.04
Mycobacterial other than tuberculosis (MOTT)	*M. intracellulare*	36.36 ± 0.41	82.14 ± 0.11
	M. terrae	36.43 ± 0.67	81.93 ± 0.10
	M. gordonae	35.19 ± 0.76	82.04 ± 0.09
	M. smegmatis	39.11 ± 0.76	82.26 ± 0.12
	M. avium	≥ 40.00	81.77 ± 0.02
	M. xenopi	35.76 ± 0.35	82.28 ± 0.44
	M. flavescens	36.84 ± 0.66	82.25 ± 0.19
	M. triviale	37.20 ± 2.40	82.62 ± 0.22
	M. kansasii	36.62 ± 0.63	82.22 ± 0.22
	M. fortuitum	35.90 ± 0.27	82.63 ± 0.14
Nonmycobacterial	*B. subtilis*	34.98 ± 0.36	82.33 ± 0.12
	Salmonella spp.	34.45 ± 0.48	82.36 ± 0.32
NTC		35.63 ± 0.84	82.08 ± 0.37

[a]Cp and [b]Tm values are based on the averages of triplicate samples (n = 3).

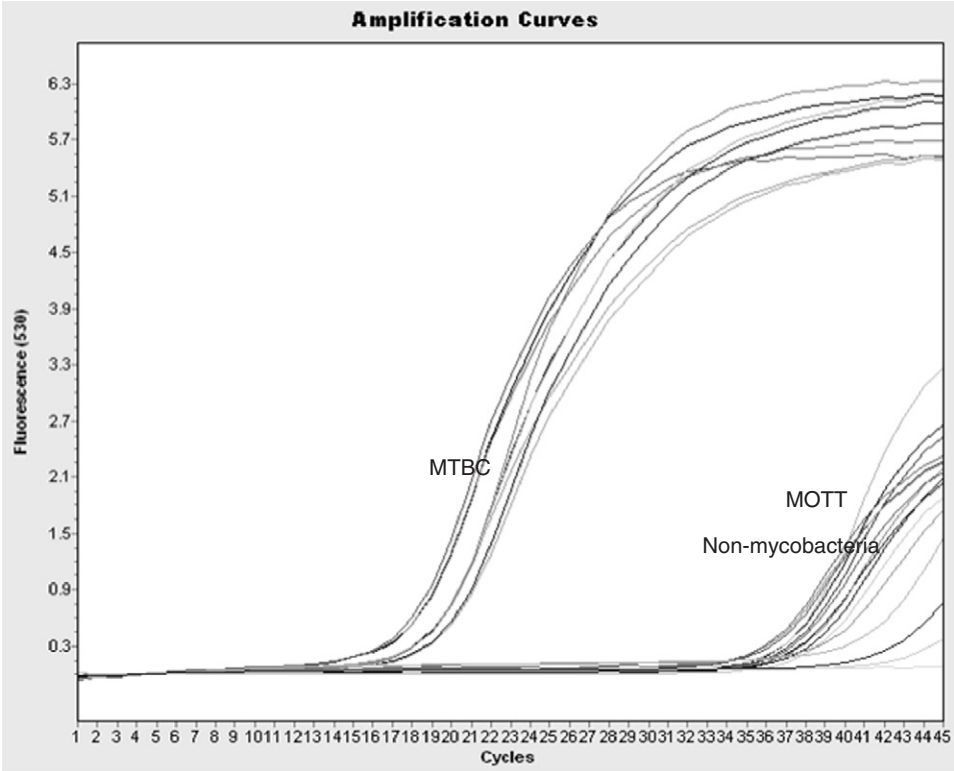

Fig. 3. Crossing points (Cp) for the detection and discrimination of MTBC (n = 3).

observed that the Cp value of 18.02 ± 0.14 cycles for the *M. tuberculosis* H37Ra was within 1 cycle (17.92 ± 0.08 cycles) of that recorded during sensitivity assay. The Tm values for MTBC were almost constant at 90.19 ± 0.06 °C, 90.27 ± 0.09 °C, and 89.81 ± 0.04 °C, respectively (Table 3, Fig. 4). The Tm analysis distinguished MTBC from MOTT.

The melting characteristics observed for MOTT, non-mycobacterial species, and NTC were due to the formation

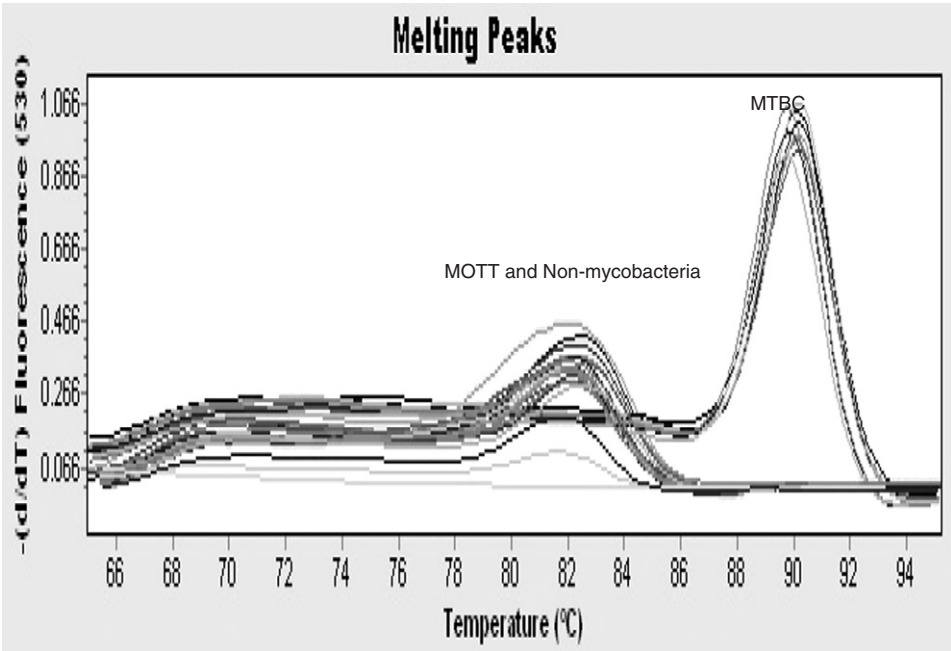

Fig. 4. Melting curve (Tm) analysis for the detection and discrimination of MTBC (n = 3).

of primer dimers. The specific products of MTBC were distinguished at higher temperature compared to the nonspecific amplicon products. Our result showed that Tm analysis of the qPCR assay can be applied for the detection and discrimination of MTBC from MOTT.

4. Conclusion

Our main objective was to develop a qPCR assay using SYBR® Green I to detect and discriminate MTBC from MOTT. The primer set used in the study was designed based on the *gyrB* gene, which has been reported as a useful phylogenetic marker for the identification of mycobacterial species. The qPCR assay is a sensitive assay because the amplification reactions displayed an equal amount of cycles for the amplification of each dilution. Tm analysis of the qPCR assay specifically showed different amplification reactions between MTBC and MOTT for the detection and discrimination of MTBC from MOTT.

The qPCR assay is currently being tested for respiratory clinical samples for the detection and discrimination of MTBC from MOTT. In the future, similar studies will be conducted with nonrespiratory clinical samples. We are currently applying probe-base and high-resolution melting (HRM) analyses. These analyses will provide specific and sensitive assays for the detection of single-base-pair difference (especially for HRM analysis) of the *gyrB* gene to differentiate among the MTBC species.

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