INTRODUCTION

Tuberculosis (TB) is a disease causing the death of approximately 1.45 million people per year worldwide among HIV-negative people and those with HIV-associated TB (WHO, 2011). The World Health Organization (WHO) and its partners have moved forward and combat dual epidemics such as TB and HIV/AIDS, and TB and diabetes (Maurice, 2011). Better diagnostic methods and therapeutic drugs are needed for screening populations for the detection of tuberculosis patients.

The Mycobacterium species are difficult to grow, requiring special expertise and equipment, or time in terms of weeks, to distinguish different mycobacteria species (Kent & Kubica, 1985). The complete nucleotide of the 16S rRNA gene and the spacer region between the 16S and 23S rRNA genes of Mycobacterium bovis BCG was previously described (Suzuki et al., 1988). The sensitivity of the primer set of the 16S rRNA gene was examined using sputum samples from patients (Pandey et al., 2008).

Several methods of nucleic acid amplification such as PCR and quantitative real-time PCR (qPCR) have been used for the detection of Mycobacterium tuberculosis. A high resolution melting (HRM) assay has been described as a nucleic acid amplification method of low cost and simplicity that provides rapid results (Reed et al., 2007); it does not need further separation steps such as gel electrophoresis (Zhou et al., 2005). The objective of this study was to demonstrate the application of the 16S rRNA-based qPCR followed by an HRM assay to detect and differentiate the closely related Mycobacterium species.

METHODS

Bacterial extraction. The Mycobacterium species used in this study were from the American Type Culture Collection (ATCC) and the 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 30 °C for Mycobacterium ulcerans and 37 °C for other Mycobacterium species. The DNA was extracted using the High Pure nucleic acid kit (Roche) according to the manufacturer’s instructions. Briefly, 200 μl of bacterial suspension was lysed, incubated at 72 °C for 15 min and centrifuged at 14 400 g.
for 1 min. Inhibitor removal buffer was added and the samples were centrifuged, washed and eluted using 50 μl of elution buffer. The eluted DNA was quantified using a NanoVue Plus spectrophotometer (GE Healthcare) according to the manufacturer’s instructions.

**Temperature gradient and primer optimization assay.** The primers F3_Avj (5′-CTG CAG GAC GAA CG-3′) and B3_Avj (5′-GCC CAT CCC ACA GTG CCG C-3′) targeting the 214 bp fragment of the 16S rRNA gene have been previously described (Pandey et al., 2008). The temperature gradient assay was performed at a range between 60 and 70 °C for assessment of the performance of the primer pair during amplification. The primer optimization assay was performed using different concentrations of the primer set (0.3, 0.4 and 0.5 μM). The 20 μl reaction volumes consisted of 10 μl 2x SsoFast EvaGreen Supermix (Bio-Rad), 1 μl 0.4 μM primers and 2 μl DNA template. The qPCR and HRM assay were performed using a CFX96 real-time PCR detection system (Bio-Rad) and results were analysed using CFX Manager and Precision Melt Analysis software (Bio-Rad). The amplification was performed using the following conditions: a pre-incubation step at 98 °C for 2 min, 45 cycles of denaturation at 98 °C for 5 s and annealing at 62 °C for 10 s, followed by the Tm analysis with increasing temperatures from 72 to 95 °C in a 0.2 °C s⁻¹ slope increment for 10 s. Results of the HRM analysis were analysed using the Precision Melt Analysis software (Bio-Rad). The clustering of the melting curves was based on the regions of the melting curve corresponding to the pre-melting, melting and post-melting regions. Distilled water was used to replace the DNA template for the non-template control (NTC) and each set of assays was performed in duplicate samples.

**Sensitivity and HRM assays.** The sensitivity assay was performed using a 10-fold dilution of the Mycobacterium avium DNA template (50 and 5 ng, 500, 50 and 5 pg) and each set of assays was performed in duplicate samples. The specificity assay was determined using 50 ng of DNA template for differentiation of closely related Mycobacterium species. In this assay, M. tuberculosis H37Rv (ATCC 27294) and H37Ra (ATCC 25177) and M. bovis IMR (M34/81) were used as species for the M. tuberculosis complex (MTBC). M. avium subsp. paratuberculosis IMR (M15/79), M. avium IPR (M170/06) and Mycobacterium intracellulare MKAK were used as species for the M. avium complex (MAC). Meanwhile, other mycobacteria species used in this assay were M. xenopi (ATCC 19250), M. smegnatis (ATCC 14468) and M. ulcerans DNA Agy 99. As the non-mycobacteria species, Bacillus subtilis (ATCC 6633) was used in the assay. Distilled water was used to replace the DNA template for the NTC, and each set of assays was performed in duplicate samples.

The HRM assay was performed in a 25 μl reaction volume containing 2 μl of template DNA, 10 μl 2x SsoFast EvaGreen Supermix (Bio-Rad) and 10 μmol of each primer of the primer set. PCR was performed using a CFX96 (Bio-Rad) thermal cycler and consisted of pre-denaturation (98 °C for 2 min) followed by 45 cycles of amplification involving denaturation (98 °C for 5 s), annealing (60 °C for 10 s) and a melting curve involving denaturation (95 °C for 30 s), annealing (70 °C for 30 s) and a melt curve (72–95 °C with an increment of 0.2 °C per 10 s). For amplification quality control, the amplified products were detected using gel electrophoresis on 1.6% agarose gels in 0.5 x Tris/ Borate/EDTA (TBE) containing red safe (3 μl per 100 ml). The gels were visualized on a UV transilluminator and photographed using a gel documentation system (Bio-Rad). Data generated were analysed using Precision Melt Analysis software (Bio-Rad). In HRM analysis, differences in Tm and normalized curve shape were used together to discriminate even the most difficult-to-detect sequence variation.

**Nucleotide sequencing and analysis.** Amplified products of the Mycobacterium species were verified using the Sanger sequencing method with the F3_Avj and B3_Avj primer set. The sequencing data were analysed against the global database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the fragment based on species. The sequence was further analysed using the multiple sequence AlignX of the Vector NTI Advance 10 (Invitrogen). A consensus sequence and guide tree of the sequencing results were observed for M. intracellulare, M. tuberculosis (H37Rv and H37Ra), M. bovis, M. smegnatis, M. xenopi, M. ulcerans, M. avium and M. avium subsp. paratuberculosis.

Accordingly a guide tree, which resembles a phylogenetic tree, was built using the neighbour-joining (NJ) method of Saitou and Nei developed in 1987. The distances are related to the degree of divergence between sequences and are displayed in the parenthesis following the mycobacteria species (Fig. 1). The 214 bp fragments of the 16S rRNA gene for the Mycobacterium species were submitted to GenBank.

**RESULTS AND DISCUSSION**

**Optimization assay**

Before proceeding with the primer optimization assay of qPCR, the annealing temperature was determined between 60 and 70 °C using the gradient temperature of the qPCR followed by the HRM analysis. The primer optimization assay of qPCR was performed for 45 cycles of amplification at the annealing temperature of 62 °C using the F3_Avj and B3_Avj primer set. The annealing temperature of 62 °C and primer concentration of 0.4 μM were used as the optimal conditions for the subsequent qPCR and HRM assay.

Confirmation of the selected conditions was determined using a 10-fold dilution starting from 50 ng to 5 pg of the M. avium DNA template. The results clearly demonstrated the need for sufficient amplification template. The quantification cycle (Cq) value for 50 pg or less of the DNA template was more than 30 cycles, whereas the Cq value for 50 ng of the DNA template was detected at 18 cycles (figure not shown). We concluded that the Cq value at above 30 cycles was accepted as a negative or inconclusive finding due to the greater variability and unreliable quantification. At different concentrations of the M. avium DNA template, the melt temperature (Tm) value was constant at 86.08 ± 0.10 °C (see Fig. S1a, available in the online Supplementary Material).

The percentage efficiency of the generated standard curve was 83.5% and the reproducibility of replicates (R²) was 0.981 (Fig. S1b). The amplification efficiency of the assay showed reliable and better quantification of the target gene compared with the previous study (Issa et al., 2012).

**Sensitivity assay**

The sensitivity of qPCR and the HRM assay were examined for the genomic DNA from mycobacteria species. Efficient amplification and melt curve analysis were observed for the Mycobacterium species. In contrast, the melt curves generated by measuring fluorescence during a temperature increase from 82.8 to 95.0 °C were not observed for the non-mycobacterial species and NTC since these formed primer dimers. The specific products of mycobacteria were distinguished at the temperature of 83.0–89.0 °C as compared to
the Tm values of the non-specific amplicon products. For each of the species included, the technical replicates clustered into the same melting pattern, thus confirming the reproducibility of the technique as previously suggested by Castellanos et al. (2010).

**Differentiation of the *Mycobacterium* species**

The application of qPCR and the HRM assay was examined for differentiation of the *Mycobacterium* species. The Tm values for *M. tuberculosis* (H37Rv, Ra) and *M. bovis* of the MTBC were 86.00 ± 0.00 °C. The Tm values for *M. avium*, *M. avium* subspecies *paratuberculosis* and *M. intracellulare* of the *M. avium* complex (MAC) were 86.20 ± 0.00 °C, 86.60 ± 0.00 °C and 86.80 ± 0.00 °C, respectively. As for other mycobacteria species, *M. smegmatis*, *M. ulcerans* and *M. xenopi*, the Tm values were 86.40 ± 0.00 °C, 86.20 ± 0.00 °C and 86.60 ± 0.00 °C, respectively.

The pre- (83.4–83.9 °C) and post-melting (88.0–88.5 °C) regions were optimized to attain the best clustering (Fig. S1a). The melting curves were normalized to eliminate the differences in background fluorescence (Fig. S2a). The normalized melting curves were shown in the form of a temperature-shifted curve along the temperature axis (Fig. 2). Accordingly, this HRM assay showed six distinct cluster patterns for the *Mycobacterium* species (Fig. S2b). The results showed that the HRM assay was able to distinguish Cluster 1 for the *M. tuberculosis* (H37Rv, Ra) and *M. bovis* of the MTBC. As for the *M. avium* and *M. avium* subsp. *paratuberculosis* of the MAC, the results showed that there was no difference between the two species assayed as there was only one cluster pattern (Cluster 2). However, based on the Tm value, a separate cluster (Cluster 6) was observed for *M. intracellulare* of the MAC. Meanwhile, it could be observed that the HRM assay produced different cluster patterns for the other mycobacteria species tested; Cluster 3 for *M. ulcerans*, Cluster 4 for *M. smegmatis* and Cluster 5 for *M. xenopi*. The differences observed in the cluster patterns were considered significant and were concordant with the consensus sequence (Fig. S3) and guide tree (Fig. 1) derived from the sequencing results. In the HRM assay as shown in Fig. 2, there was no distinct difference for *M. tuberculosis* (H37Rv and Ra) and *M. bovis*. Hence, a difference of two bp as observed in the consensus sequence of MTBC did not result in a different cluster pattern for *M. bovis* as compared to *M. tuberculosis* (H37Rv and Ra). However, it is in agreement with the grouping of MTBC using biochemical tests for differentiation of the *Mycobacterium* species. In concordance with the consensus sequence and guide tree of the MAC, the HRM assay produced a different cluster pattern for *M. avium* and *M. avium* subsp. *paratuberculosis* compared with *M. intracellulare*.

It can be seen from the observed cluster patterns that the HRM assay is specific, sensitive and able to differentiate species based on small sequences as suggested by Garritano et al. (2009). A previous study on *M. avium* subsp.
**Differentiation of Mycobacterium species of* Mycobacterium* species**

![Fig. 2. HRM assay of the temperature-shifted difference curves of the amplified 16S rRNA gene of the* Mycobacterium* species. Each trace represents a replicate (n=3). RFU, Relative fluorescence units.](image)

*paratuberculosis* types I, II and III was reported by Castellanos et al. (2010). The results showed that in this study, the cluster patterns of the HRM assay can be applied for differentiation of the *Mycobacterium* species. Therefore, although some species that are frequently present in clinical specimens such as *M. fortuitum*, *M. chelonae* and *M. kansasii* were not investigated, it is hoped that this study could indicate the importance of HRM analysis for the differentiation of *Mycobacterium* species which has not been previously reported.

The detection of TB using the 16S rRNA gene in a molecular method such as the *M. tuberculosis* loop-mediated isothermal amplification (LAMP) system is a rapid and sensitive method (Pandey et al., 2008) but limited in its ability to identify the infecting *Mycobacterium* species. This study, using the 16S rRNA gene as the target in the qPCR followed by the HRM assay, is a sensitive assay showing distinct cluster patterns for the different *Mycobacterium* species as previously suggested by Erali et al. (2008).

In conclusion, this study showed that the qPCR using the 16S rRNA gene as the target followed by the HRM assay can be applied in the laboratory. The observed distinct cluster patterns of the HRM assay allow for prompt identification of *Mycobacterium* species. This assay will provide a much needed method for early laboratory diagnosis.

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**REFERENCES**


