CHAPTER 5

Development of a New Alternative Diagnostic Tool using Real-Time PCR Coupled with High Resolution Melting (HRM) Analysis Targeting the Second Internal Transcribed Spacer (ITS-2) of Nuclear Ribosomal DNA

5.1 INTRODUCTION

Hookworms are one of the most common parasitic nematodes that inhabit the small intestine of humans and other mammal such as canine and feline. The two primary species of hookworm infecting humans are *Ancylostoma duodenale* and *Necator americanus* (Chan et al, 1994a), with *A. duodenale* occurring mainly in the Middle East, North Africa, India, Australia and Europe, whilst *N. americanus* in the Americas, Sub-Saharan Africa, East Asia and Southeast Asia (de Silva et al, 2003). The socioeconomic and public health impact of human hookworm infections are extensive, infecting an estimated 600 million people worldwide and resulting in up to 135,000 deaths annually (de Silva et al, 2003; Anon, 2005; Bethony et al, 2006).

The gravest consequences are manifested in children and women of childbearing age (de Silva et al, 2003) displaying chronic intestinal blood loss which may result in anaemia, iron deficiency anaemia (IDA) and protein malnutrition. The most deleterious effects of hookworm infections include impaired physical, intellectual and cognitive development of children, increased mortality in pregnant women and their infants and reduced work capacity of adolescents and adults (Albonico et al, 1999; Crompton, 2000; Crompton & Nesheim, 2002; Anon, 2002; 2005). Besides the two anthropophilic species of human hookworm (i.e., *N. americanus* and *A. duodenale*), canine and feline hookworm (e.g., *Ancylostoma ceylanicum*, *Ancylostoma braziliense* and *Ancylostoma*

caninum) are also able to cause zoonotic disease to humans such as creeping eruption and eosinophilic enteritis (EE) or less frequently symptoms including localized myositis, erythema multiforme or ophthalmological manifestations (Bowman et al, 2010).

Accurate diagnosis and genetic characterization of hookworms by precise identification and differentiation of species involved is essential in monitoring the efficacy of mass treatment and effective control measures. Currently, most research conducted on the epidemiology of hookworm and other intestinal nematodes have relied on the use of conventional microscopy for the identification of eggs in feces and third-stage larvae (L3) through the coproculture technique (Dunn & Keymer, 1986; Hata et al, 1992). The benefits of this method are mainly due to technical simplicity and low cost. However, utilization of microscopy is limited by the fact that hookworm eggs are morphologically indistinguishable from those of other strongylid nematodes (e.g., *Trichostrongylus* spp. and *Oesophagostomum* spp.) and it is laborious, time-consuming and requires relatively skilled personnel (Jozefzoon & Oostburg, 1994).

Frequently, mass treatment with anthelminthic drugs is performed without the identification of the causative species of infection. Given that clinical manifestation such as severity of anaemia differs according to the hookworm species involved (Beaver et al, 1984) and the route of infection for each hookworm species also differ from each other, for example *N. americanus* infection is mainly by skin penetration, while the ingestion of infective third stage larvae is more common for *Ancylostoma* spp. infections, hence, species identification is paramount in designing appropriate and effective prevention and control strategies. Moreover, if a zoonotic hookworm is prevalent, the control target and strategies formulated need to encompass animal hosts as well. Thus, there is a crucial need for a practical, highly sensitive and specific

diagnostic and analytical tool, particularly one based on the polymerase chain reaction (PCR) (Gasser, 2006a) to address key epidemiology and population genetic questions to underpin surveillance, treatment and control program.

Following extensive evaluation of the specificity of genetic markers of hookworm such as first (ITS-1) and second (ITS-2) internal transcribed spacer of nuclear ribosomal DNA (rDNA), several techniques have been developed for the identification and characterization of hookworm at the molecular level. These include conventional and semi-nested PCR (Gasser et al, 1993; Romstad et al, 1997; Chilton & Gasser, 1999; Verweij et al, 2001; de Gruijter et al, 2005a) and single-strand conformation polymorphism (SSCP) (Gasser et al, 2006b), mutation scanning (Gasser et al, 1998), PCR-restriction fragment length polymorphism (RFLP) (Hawdon, 1996; Traub et al, 2004) and amplified fragment length polymorphism (AFLP) or random amplification of polymorphic DNA (RAPD) (de Gruijter et al, 2005b; 2006). Although these approaches are very useful and effective, the electrophoretic analysis can be quite time consuming to perform. Moreover, the amplification and detection of DNA are prone to false-positive results due to cross contamination and are expensive, with the endpoint reading on agarose gels yielding no quantitative information. Additionally, multiplex real-time PCR using fluorescent detection probes through the possibility of combining assay for the detection of different target into one reaction has been developed for the diagnosis of hookworm infection in humans (Verweij et al, 2007; Basuni et al, 2011), however this technique is relatively expensive.

Due to the increased demand for rapid, high-throughput diagnosis and genetic analysis of pathogens as well as data handling and analysis, there has been a considerable focus on the evaluation and development of advanced detection methods which obviate the need for electrophoretic analysis, reduce the risk of contamination

and substantially decrease labor time and reagent costs. For example, the introduction of high-resolution melting (HRM) analysis coupled with real-time PCR assay which is a relatively new post-PCR analysis that allows direct characterization of PCR amplicons in a closed system (Wittwer et al, 2003). Probe-free HRM real-time PCR assay does not require the multiplex method, has no manual post-PCR processing, is performed in a closed-tube system and has a low reaction cost relative to other methods for rapid screening and detection of closely related species in a laboratory. Such approach simply generates DNA melt-curve profiles which are sufficiently sensitive and specific for genotyping and species differentiation based on their unique and distinct melting patterns (Wittwer et al, 2003; Montgomery et al, 2007).

To date, the HRM has mostly been used in human clinical studies (Gundry et al, 2003; Wittwer et al, 2003; Liew et al, 2004; 2006; Zhou et al, 2004; Chou et al, 2005; Dobrowolski et al, 2005; Palais et al, 2005; Willmore-Panye et al, 2005; 2006; Cheng et al, 2006; Prathomtanapong et al, 2009; Radvansky et al, 2010; Saitsu et al, 2010). In addition, it also has been used in microbial studies, for example in species identification of *Aspergillus* spp. (Erali et al, 2006), *Salmonella* spp. (Slinger et al, 2007) and *Staphylococcus* spp. (Lilliebridge et al, 2011). However, the application of the HRM technique to the diagnosis of parasitic organisms has been rather limited and the method has mostly been applied in molecular studies of parasitic protozoa such as the differentiation of old world *Leishmania major* from *Leishmania donovani* or *Leishmania tropica* or *Leishmania infantum* and *Leishmania aethiopica* infecting humans (Nicolas et al, 2002; Nasereddin & Jaffe, 2010; Talmi-Frank et al, 2010). Likewise, Pangasa and co-workers (2009) distinguished three *Cryptosporidium* spp. (i.e., *Cryptosporidium hominis*, *Cryptosporidium parvum* and *Cryptosporidium meleagridis*) based on their melting profiles in human clinical samples. Similarly, HRM

technique has been successfully used to differentiate *Plasmodium* spp. infecting humans (i.e., *Plasmodium falciparum, Plasmodium ovale* and *Plasmodium malariae*) in human clinical samples (Mangold et al, 2005; Andriantsoanirina et al, 2009). Such approach has also been applied in other protozoa studies including *Dientamoeba fragilis* (Hussein et al, 2009), *Naegleria* spp. (Robinson et al, 2006) and *Giardia* spp. (Bienz et al, 2001). As for parasitic worm, the application of the HRM method has been rather sporadic. The technique has been used for rapid differentiation of *Brugia malayi* and *Brugia pahangi* (Areekit et al, 2009) and population studies of *Fascioloides magna* (Radvansky et al, 2011). By extending such approaches on parasitic nematode worms with regards to hookworm, the present study attempt to evaluate the possible use of real-time PCR coupled with high resolution melting (HRM) analysis for the rapid identification, quantification and differentiation of hookworm species as a reliable alternative to traditional microscopy, conventional PCR or probe-based genotyping tools.

5.1.1 Objectives of the study

General objective

To develop a new alternative diagnostic tool for the rapid detection, quantification and speciation of hookworm species in human samples using real-time PCR coupled with high resolution melting (HRM) analysis targeting the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA as genetic marker.

Specific objectives

1. To establish a practical PCR-coupled melting curve analysis for the accurate identification and differentiation of hookworm species infecting humans based on their unique and distinct melting profiles.

2. To evaluate the specificity and sensitivity of this approach in comparison with the traditional microscopy and conventional PCR assay.

3. To determine the species of hookworm infecting humans in Malaysia.

4. To discuss the implications of this approach as an alternative molecular diagnostic tool for the dynamic surveys and epidemiological studies of hookworm infections.

5.1.2 Research hypotheses

1. A new alternative diagnostic tool using real-time PCR-HRM analysis will be developed for a rapid and accurate detection and identification of hookworm species infecting humans in Malaysia.

2. The most common hookworm species in this region (i.e., *N. americanus*) is expected to be found in human fecal sample. Meanwhile, *A. duodenale* is unlikely to be detected in accordance to their geographic restriction in this

region. In addition, animal hookworm especially *A. ceylanicum* is also likely to infect human.

3. The sensitivity and specificity of this assay is expected to be improved compared to microscopy and conventional semi-nested PCR.

5.1.3 Significance of the study

Hookworm infections are most prevalent in the bottom billion of the world's poorest people especially in tropical and subtropical countries. To date, approximately 600 million people in the world are infected. Besides the two anthropophilic species infecting humans (i.e., *N. americanus* and *A. duodenale*), animal hookworms such as *A. ceylanicum, A. caninum* and *A. braziliense* can also cause infections in humans, with significant geographic overlap in their distributions. Basically, hookworm diagnosis still relies on microscopic examination due to its technical simplicity and low cost. However, this technique is limited by the fact that hookworm eggs of different species are morphologically indistinguishable. Recently, several probe-based genotyping assays have been developed for the identification of hookworm species at the molecular level. Although these approaches are very useful and effective, they are also laborious and time consuming especially the post-PCR processing steps, pose higher risk of contamination, expensive and may only provide qualitative information. Thus, there is a crucial need for a simple and rapid technique such as the method reported in this study that can serve as an alternative analytical tool for improved diagnosis and surveillance of hookworm infections.

5.2 MATERIALS AND METHODS

5.2.1 Background

Details of the consent, sample collection, sampling scheme, population sampled and microscopic examination of fecal samples prior to this work have been previously described in **Chapters 3**.

5.2.2 Control, specificity and sensitivity test

To establish the real-time PCR coupled with high resolution melting (HRM) assay, a well-defined hookworm genomic DNA was obtained from fecal samples in which *N. americanus*, *A. ceylanicum* and *A. caninum* were confirmed by the conventional PCR technique. Due to limited positive controls for other species, genomic DNA of *A. duodenale* and *A. braziliense* were isolated from individual adult worms (kindly supplied by Dr. Megumi Sato from Niigata University, Japan). The assay's specificity was evaluated using a set of control DNA standards including DNA from a fecal sample of an individual with no history of parasitic infections and DNA from other parasitic nematodes (i.e., *Ascaris lumbricoides, Trichuris trichiura, Strongyloides stercoralis* and *Trichostrongylus* spp.) and protozoa (i.e., *Giardia lamblia, Cryptosporidium* spp., *Blastocystis* spp., *Entamoeba histolytica, Entamoeba dispar* and *Entamoeba moshkovskii*). Since the number of parasite genomes present in each sample cannot be accurately determined, the lowest detectable concentration (i.e., sensitivity of the assay) was assessed using 10-fold serial dilutions of 10 ng/µl positive control of the genomic DNA, ranging from 10^{-1} to 10^{-5}.

5.2.3 Genomic DNA extraction

Prior to DNA amplification, genomic control DNA for the different types of intestinal parasites and also human fecal samples infected with hookworm were extracted using the PowerSoil® DNA Kit (MO BIO, cat. no. 12888-100, CA, USA) according to manufacturer's instructions (Appendix C). Briefly, approximately 0.2 to 0.3 g of fecal pellet was added into the PowerBead Tube®, incubated at 70ºC for 10 minutes with the presence of cell lysis and disruption agent provided in the kit. Subsequently, the fecal samples were subjected to homogenization and lysis procedures for complete cell lysis by mechanical shaking (vortexing) using MO BIO Vortex Adapter (MO BIO, cat. no. 13000-V1). Final elution of DNA was made in 50 µl of elution buffer instead of 200 µl. As for individual adult worms, genomic DNA was extracted using the QIAamp® DNA Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions (Appendix C). In brief, individual worms were suspended in 180 ATL tissue lysis buffer (QIAgen, Hilden, Germany) before being treated with sodium dodecyl sulfate-proteinase K followed by incubation at 56°C for 1 hour. The purity and concentration of extracted DNA was qualitatively measured using a NanoPhotometer (IMPLEN, Germany). Extracted DNA was then stored at -20°C until required for PCR amplification.

5.2.4 Real-time PCR-HRM primer designing

Briefly, the primers were designed specifically to amplify all five hookworm species (i.e., *N. americanus, A. duodenale, A. ceylanicum, A. caninum* and *A. braziliense*) from previously published sequences in GenBank (accession numbers AF217891,

EU344797, DQ438080, EU159416 and DQ438064). The published sequences of the five hookworm species were then manually aligned and edited to obtain the consensus sequence using the BioEdit Sequence Alignment version 7.0.9 program (Hall, 1999) and CLUSTAL-W analysis (Thompson et al, 1994) (Appendix D). Subsequently, a single pair of degenerate primers were designed separately with the aide of sequence analysis and Primer Express software (Applied Biosystems, Inc., CA, USA), followed by *in silico* PCR analysis as previously described (Thong et al, 2011, Ng et al, 2012) to ensure the designed primers were targeting to the genomic region of interest before forming the desired degenerate primers. In addition, the designed primers were also checked through the Basic Local Alignment Search Tool (BLAST) hosted by National Centre for Biotechnology Information (NCBI) (Bethesda, MD) to ensure no nonspecific amplification from non target DNA. Finally, a pair of degenerate primers UMF (Forward: 5'-CACTGTTTGTCGAACGGYAC-3') and UMR (Reverse: 5'-AGTCSVKRRRCGATTMARCAG-3') were designed to amplify approximately 180-200 bp within the 5.8S and second internal transcribed spacer (ITS-2) ribosomal RNA region of the hookworm species.

5.2.5 Preliminary optimization of the primer using conventional PCR

For preliminary optimization of the primers, a series of gradient PCR assays using conventional PCR was carried out using a wide range of isolated DNA (i.e., non-infected humans, hookworms and DNA from other intestinal parasites) in order to obtain the optimal annealing temperature for the primers. Briefly, the PCR was carried out using 50 µl of PCR mixture containing 10x PCR buffer (New England Biolabs, cat. no. M0267S, Ipswich, USA), 1.25 mM dNTPs (Fermentas, cat. no. R0193, Ontario,

Canada), 4 mM $MgCl_2$ (Fermentas, cat. no. R0193, Ontario, Canada), 10 pmole of each primer, 1 U of *Taq* polymerase (New England Biolabs, cat. no. M0267S, Ipswich, USA) and 6 µl of DNA template. The sample was heated at 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds (denaturing), 50°C to 60°C for 30 seconds (gradient annealing temperature), 72°C for 30 seconds (extension) and a final extension at 72°C for 7 minutes. DNA blank (DNase/RNase free water, Sigma Cat. no. W4502, St. Louis, MO) and positive genomic DNA were also included during each PCR optimization. Cycling was performed in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA).

5.2.6 Real-time PCR-HRM assay

Upon completion of the primer optimization using conventional PCR, real-time PCR was performed in a total reaction mixture of 20 µl containing 10 µl of MeltDoctor HRM Master Mix (Applied Biosystems, Inc., CA, USA), 10 pmole of each primer, approximately 10 ng/µl of genomic DNA and sterile deionized water using a 7500 Fast real-time PCR system (Applied Biosystems, Inc., CA, USA). Genomic DNA of positive controls (hookworm) and control samples without DNA (DNase/RNase free water, Sigma Cat. no. W4502, St. Louis, MO) were included in each PCR run. The PCR thermocycling conditions was set according to the optimized protocol at 95°C for 10 minutes (enzyme activation) followed by amplification for 40 cycles consisting of 95°C for 15 seconds (denaturation step) and 60°C for 1 minute (annealing and elongation steps).

Following the real-time PCR, amplicon dissociation was immediately started by a melting step in the same real-time PCR machine. The program consisted of

denaturation at 95°C for 10 seconds, 57°C for 1 minutes (annealing), 95°C for 15 seconds (high resolution melting) and final annealing at 60°C for 1 minutes. In this process, the PCR amplicons were allowed to denature and re-anneal before the high resolution melting recording changes in fluorescence with changes in temperature (dF/dT) and plotting against changes in temperature (Appendix E).

The high resolution melting curve profile was then analyzed using Applied Biosystems High Resolution Melting (HRM) software version 2.0.1 (Applied Biosystems, Inc., CA, USA) with fluorescence (i.e., melting curve) normalization by selecting the linear region before and after the melting transition. Melting temperature (T_m) was interpolated from the normalized data as the temperature at 50% fluorescence. Different species were easily distinguished by plotting the fluorescence difference between normalized melting curves. All samples of hookworm species (i.e., control) were examined in triplicate to obtain the standard deviation (SD) for the melting temperature (T_m).

5.2.7 Conventional semi-nested PCR

5.2.7.1 DNA amplification

A two-step semi-nested conventional PCR was used for DNA amplification of hookworm species in 634 human fecal samples. For the first amplification, the forward primer NC1 (5’-ACG TCT GGT TCA GGG TTC TT-3’) and reverse primer NC2 (5’-TTA GTT TCT TTT CCT CCG CT-3’) (Gasser et al, 1993) were used to amplify an approximately 310 bp and 420 bp regions of internal transcribed spacer-2 (ITS-2) and 28S ribosomal RNA region of *N. americanus* and *Ancylostoma* spp. The PCR was

carried out in a 50 µl final mixture containing 10x PCR buffer (New England Biolabs, cat. no. M0267S, Ipswich, USA), 2.5mM dNTPs (Fermentas, cat. no. R0193, Ontario, Canada), 25 mM $MgCl_2$ (Fermentas, cat. no. R0193, Ontario, Canada), 10 pmole of each primer, 5U of *Taq* polymerase (New England Biolabs, cat. no. M0267S, Ipswich, USA) and 6 µl of DNA template. The sample was heated to 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds (denaturing), 55°C for 30 seconds (annealing), 72°C for 30 seconds (extension) and a final extension at 72°C for 7 minutes. Control samples without DNA (DNase/RNase free water, Sigma Cat. no. W4502, St. Louis, MO) and samples containing *N. americanus* and *Ancylostoma* spp. genomic DNA (i.e., positive control) was included in each PCR.

Subsequently, samples that produced fragment approximately 310 and/or 420 bp in the first PCR were subjected to a second amplification. Amplification was conducted by using forward primer NA (5'-ATGTGCACGTTATTCACT-3') for *N. americanus* (Verweij et al, 2001) and AD1 (5'- CGA CTT TAG AAC GTT TCG GC-3') for *Ancylostoma* spp. (de Gruijter et al, 2005a) and NC2 as a common reverse primer to produce a 250 and/ or 130 bp amplicon that corresponded to *N. americanus* and *Ancylostoma* spp., respectively. The secondary amplification reagent concentrations were similar to those of the first round of PCR except that 6 µl of primary PCR product (i.e., NC1-NC2 amplicon) was added instead of DNA. The cycling conditions for the second round amplification were 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minutes (denaturing), 55°C for 1 minutes (annealing), 72°C for 1 minutes (extension) and a final extension at 72 °C for 7 minutes. In both amplifications, samples were incubated in the MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA).

All PCR amplicons were subjected to electrophoresis in 2% (w/v) agarose gels at 100V for 35 min and visualized in ultraviolet transilluminator after staining with SYBR Safe DNA stain (Invitrogen, USA). Briefly, 5 µl of PCR amplicon was mixed with 3 µl of 6x loading dye (Fermentas, cat. No. SM0241, Ontario, Canada) on a small sheet of clean parafilm (PARAFILM, USA). The mixture was then transferred into the well set in the agarose gel prior to gel electrophoresis (Appendix F).

5.2.7.2 Identification of hookworm species

Positive amplicon generated from each sample were then purified using the QIAquick PCR Purification Kit (QIAgen, cat. no. 28104, Hilden, Germany) according to the manufacturer's instructions (Appendix G). The final elution of DNA was made in 30 µl of elution buffer instead of 50 µl. All purified amplicons were subjected to DNA sequencing in both directions using a BigDye® Terminator v3.1 sequencer (Applied Biosystems, Inc., CA, USA). Sequence chromatograms were viewed using Sequence Scanner version 1.0 (Applied Biosystems, Inc., CA, USA). Both forward and reverse sequences were manually aligned and the consensus sequence was created for each sample using the BioEdit version 7.0.9 (Hall, 1999). Homology search was conducted using National Centre for Biotechnology Information (NCBI) (Bethesda, MD) reference sequences with the Basic Local Alignment Search Tool (BLAST) as the means to determine hookworm species. All sequences generated were deposited in NCBI GenBank (Appendix H).

5.2.8 Screening of human fecal samples using real-time PCR-HRM assay

Before this approach was to be applied for the screening of all studied human fecal samples, confirmation of the five hookworm species which served as positive controls was done on the basis of a homology search using BLAST program hosted by NCBI reference sequences following similar protocol as previously described in **Chapter 5.2.7.2 (Identification of hookworm species)**. Briefly, two randomly selected positive amplicons of each species derived from real-time PCR-HRM that displayed distinct curve shapes and T_m were purified and subjected to DNA sequencing. Subsequently, a total of 634 human fecal samples regardless of their microscopy results were then subjected to DNA amplification using the real-time PCR-HRM assay for the identification of hookworm species.

5.2.9 Comparison between real-time PCR-HRM assay, conventional semi-nested PCR and microscopy

The performance (i.e., sensitivity and specificity) of the real-time PCR-HRM assay was also validated by comparing the obtained results from the real-time PCR-HRM assay with the microscopy **(Chapter 3)** and conventional semi-nested PCR **(Chapter 5.2.7)** results.

5.3 RESULTS

5.3.1 Specificity and sensitivity of the primer, PCR condition and amplicon

The specificity of the primer was examined using various genomic DNA from human feces (i.e., negative for parasitic infection) and samples positive with a range of intestinal parasites (i.e., both intestinal helminth and protozoa) prior to microscopy and conventional PCR examination. In this assay, only the amplification and HRM plots of positive controls (i.e., hookworm DNA) was detected while no amplification of other genomic DNA (i.e., in human feces negative for parasitic infections or samples of DNA from protozoa or other helminths) was observed. Subsequently, the amplicons derived from selected genomic samples representing hookworm DNA (i.e., positive control) and other DNA samples were verified by 2% (w/v) gel electrophoresis. On agarose gels, only the amplicon from hookworm DNA (approximately 180-200 bp) was observed while no bands were detected for other parasite DNA samples (Figure 5.1). The amplicons from randomly selected samples representing all five hookworm species were sequenced for species conformation based on sequence comparison using BLAST with reference sequences from GenBank shown to represent *N. americanus* (accession number JF960390), *A. duodenale* (accession number EU344797), *A. ceylanicum* (accession number JN120876), *A. caninum* (accession number JN120895) and *A. braziliense* (accession number JF120898), respectively.

Given that the copy number of parasite genomic DNA present in each sample cannot be accurately determined, the sensitivity was assessed by using a well-defined reference DNA control to determine the lowest detectable DNA concentration in this assay. The assay sensitivity was assessed by using 10-fold serial dilutions of 10 ng/µl of

predefined hookworm genomic DNA (i.e., ranging from 10^{-1} to 10^{-5}). No amplification was noted at the dilution of 10^{-4} and 10^{-5}, and therefore 10^{-3} (0.01 ng/µl) marked the lowest dilution at which parasite DNA was detected (Figure 5.2).

5.3.2 Sample categorization based on HRM curve profile

In order to examine the reproducibility (i.e., consistency) of each melting profile, amplicons representing the reference control DNA from each hookworm species were tested in triplicate and repeated on several different days by keeping the same chemistry environment with similar reagents and DNA concentrations. Our results demonstrated that the reproducibility of the assay was very high with consistent melting patterns between runs for each species analyzed on different days. The melting characteristics of ITS-2 amplicons from all species were assessed by plotting three different curves (Figure 5.3 - 5.5). In the present study, the normalized fluorescence curves (i.e., aligned melt curve) (Figure 5.3), derivative melt curve (Figure 5.4) and difference plot melt curve (Figure 5.5) produced uniquely different plots that were easily distinguishable for each species. The melting curves were characterized by peaks of 79.24 ± 0.05°C and 83.00 ± 0.04°C in profile 1 (*N. americanus*), 79.12 ± 0.10°C in profile 2 (*A. duodenale*), 79.40 ± 0.10°C in profile 3 (*A. ceylanicum*), 79.63 ± 0.05°C in profile 4 (*A. caninum*) and 79.70 ± 0.14°C in profile 5 (*A. braziliense*) (Table 5.1). As for *Ancylostoma* spp., although their melting profiles (T_m) were almost similar to each other, they could clearly be discerned by the plotting of normalized melting curves (Figure 5.3) and temperature-shifted fluorescence difference (Figure 5.5).

For each of the hookworm species, a sharp decreased in fluorescence was detected in denatured DNA as shown in normalized fluorescence curves (Figure 5.3),

which was consistent with its respective melting profile (Figure 5.4). It was also noted that this assay easily distinguished between *N. americanus* (profile 1) and *Ancylostoma* spp. (profiles 2 to 5) by the presence of two peaks in profile 1 compared to a single peak in the other profiles representing *Ancylostoma* spp. as demonstrated in the derivative melt curve (Figure 5.4).

5.3.3 Identification of hookworm species in human fecal samples using real-time PCR-HRM assay

Details on the prevalence of hookworm infections in humans based on microscopy examination have been previously described **(Chapter 3)**. In brief, a total of 9.1% (58/634; 95% CI=7.0-11.7%) human fecal samples were microscopically positive with hookworm-like eggs. The optimized real-time PCR coupled HRM assay targeting the ITS-2 region of *Necator americanus* and *Ancylostoma* spp. was then used to screen and identify hookworm species in these human fecal samples. Of the 58 microscopically positive-hookworm fecal samples, all (58/58; 100%) were successfully characterized on the basis of its unique and distinct characteristics of their melting curve patterns (Table 5.2). In order to avoid false positive results via microscopy, all microscopically negative human fecal samples (i.e., 576 samples) for hookworm infection were also subjected to the same amplification procedure. However, none of these samples were detected positive for hookworm infections.

Overall, our real-time PCR-HRM assay results demonstrated that 89.6% (52/58) and 19.0% (11/58) were *N. americanus* and *A. ceylanicum*, respectively. Of this, 81.0% (47/58) and 10.3% (6/58) of humans had single infections with *N. americanus* and *A. ceylanicum*, respectively and 8.6% (5/58) had mixed infections with both species (Table

5.3). The highest prevalence of *N. americanus* was recorded in Gurney village (25/58; 43.1%), followed by Pos Iskandar (13/58; 22.4%), Bukit Serok (8/58; 13.8%), Kuala Pangsun (4/58; 6.9%) and the lowest prevalence was recorded in Sungai Bumbun (1/58; 1.7%). As for *A. ceylanicum*, 8.6% (5/58) were detected in persons in Gurney village, 3.4% (2/58) in Pos Iskandar and Kuala Pangsun, and 1.7% (1/58) in Bukit Serok and Sungai Bumbun, respectively.

5.3.4 Identification of hookworm species in human fecal samples using conventional semi-nested PCR

Out of 58 microscopically positive human fecal samples, 81.0% (47/58) were successfully amplified and genetically characterized using conventional semi-nested PCR on the basis of its DNA sequence (Table 5.2). Of this, sequence comparison using BLAST demonstrated that 87.2% (41/47) and 23.4% (11/47) were *N. americanus* and *A. ceylanicum*, respectively. Of this, 76.6% (36/47) and 12.8% (6/47) of humans harbored single infections with *N. americanus* and *A. ceylanicum* respectively, while 10.6% (5/47) had mixed infections with both species. Similarly, none of the microscopically negative-hookworm samples were detected positive for hookworm infections when subjected to the same amplification (data not shown).

5.3.5 Comparison between real-time PCR-HRM assay, conventional semi-nested PCR and microscopy

The comparison between microscopy, conventional semi-nested PCR and the real-time PCR-HRM assay was also explored in order to validate the performance of our real-

time PCR-HRM assay (Table 5.4). Fifty-eight out of 634 fecal samples were microscopically positive for hookworm-like eggs. However, specific hookworm amplification was only detected in 47 (81.0% of 58) samples via conventional semi-nested PCR in which hookworm-like eggs were seen in the iodine stained direct smear examination. The same fecal samples (N=634), regardless of the infection status, were also analyzed for species identification by HRM real-time PCR. In our real-time PCR-HRM assay, specific amplification was detected in all 58 (100%) samples in which hookworm-like eggs were seen by microscopy. No amplification was detected in 576 microscopically negative samples by both the conventional semi-nested PCR and the HRM real-time PCR assays leaving the true prevalence of the hookworm infections in the studied population as 10.1% (58 of 634).

The sensitivity and the specificity of the conventional semi-nested PCR and real-time PCR-HRM assays for detection of hookworm infections were also evaluated in the present study. As for the specificity (i.e., the ability of the assay to identify true positive hookworm infections), both assays gave 100% specificity (Figure 5.6). With regards to the sensitivity (i.e., the ability of the assay to identify true negative hookworm infections), our real-time PCR-HRM assay (100%) had higher sensitivity as compared to conventional semi-nested PCR (84.1%) (Figure 5.6). In our conventional semi-nested PCR assay, we were not able to amplify 11 samples in which hookworm-like eggs were seen via microscopy; however, these samples were amplified and identified as *N. americanus* based on their melting profile by the HRM assay (Figure 5.7). In addition, five mixed infections of *N. americanus* and *A. ceylanicum* detected in our conventional semi-nested PCR assay also produced a unique melting point plot that was easily distinguishable from single infection cases via our real-time PCR-HRM assay (Table 5.5).

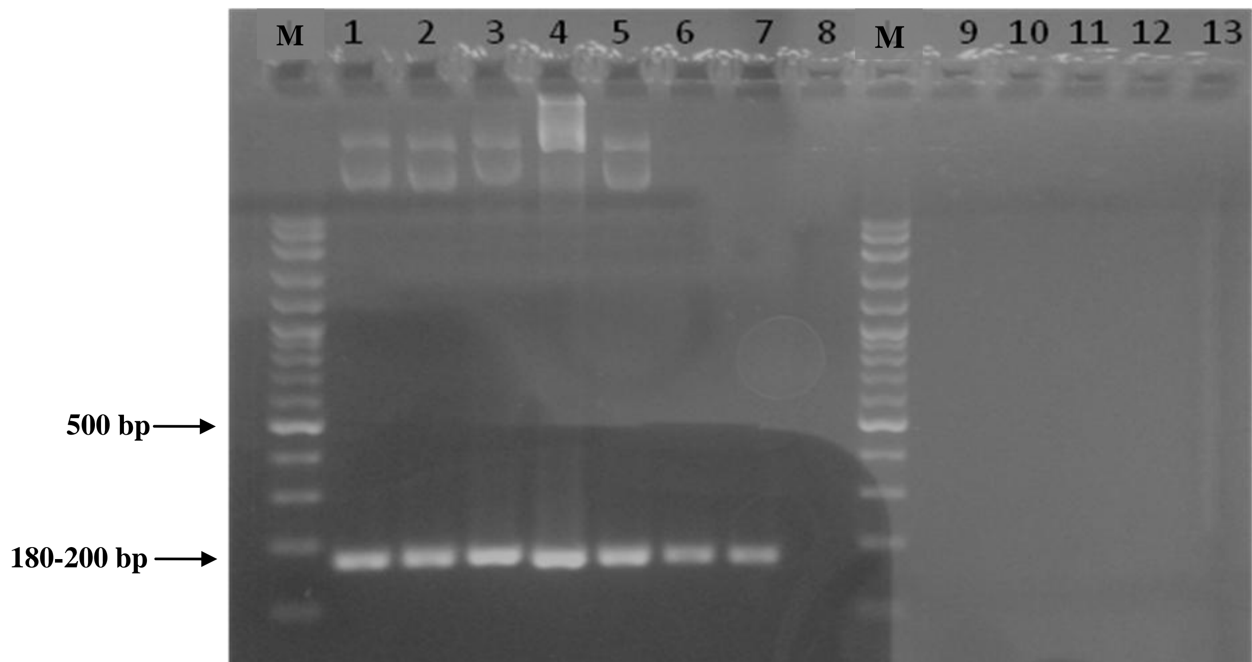

Figure 5.1: Representative of the amplicons derived from selected positive controls verified by 2% (w/v) gel electrophoresis

Legend:

M = 100-basepair DNA marker, with molecular size indicated in base-pair unit for prominent band

Lane 1 to 5 = Genomic DNA for each hookworm species

Lane 6 = Positive control (Genomic DNA of hookworm extracted from adult worm)

Lane 7 = Positive control (Genomic DNA of hookworm extracted from microscopically positive fecal sample)

Lane 8 = Negative control (DNase/RNase free water)

Lane 9 to 13 = Genomic DNA for the representative of other intestinal helminth and protozoa

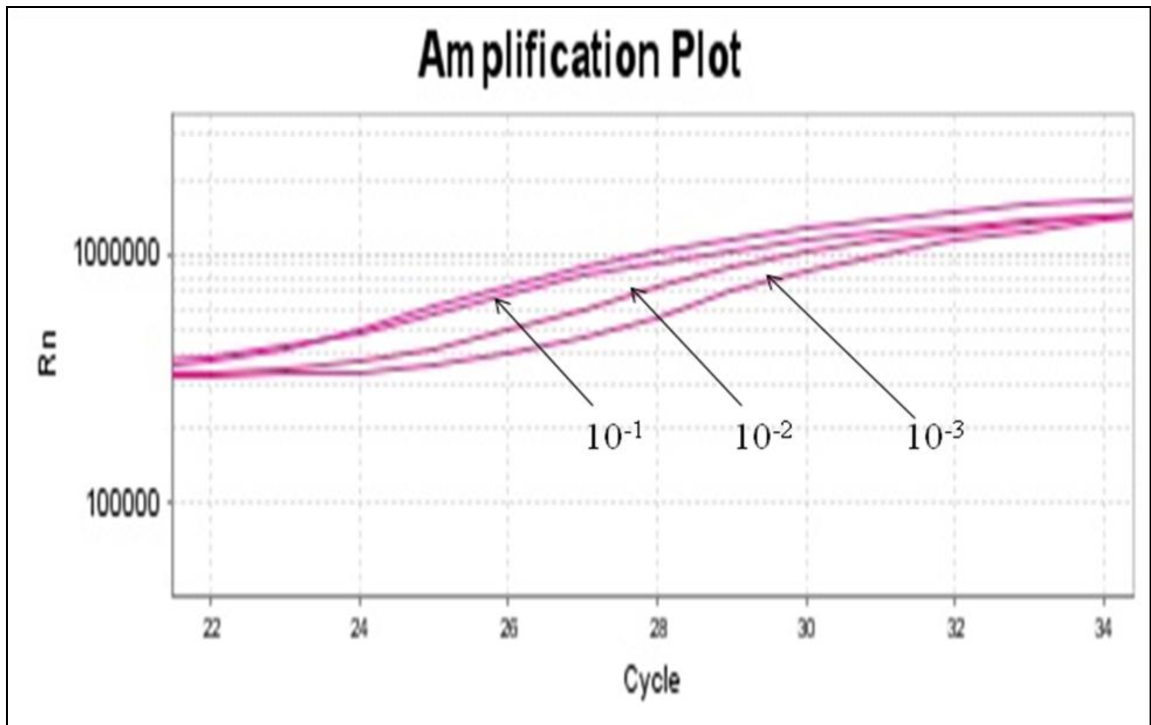

Figure 5.2: Sensitivity test by using 10-fold serial dilutions of well defined reference control DNA. No amplification was noted after dilution of 10^{-3}, therefore 10^{-3} (0.01) marked the lowest dilution at which parasite DNA can be detected

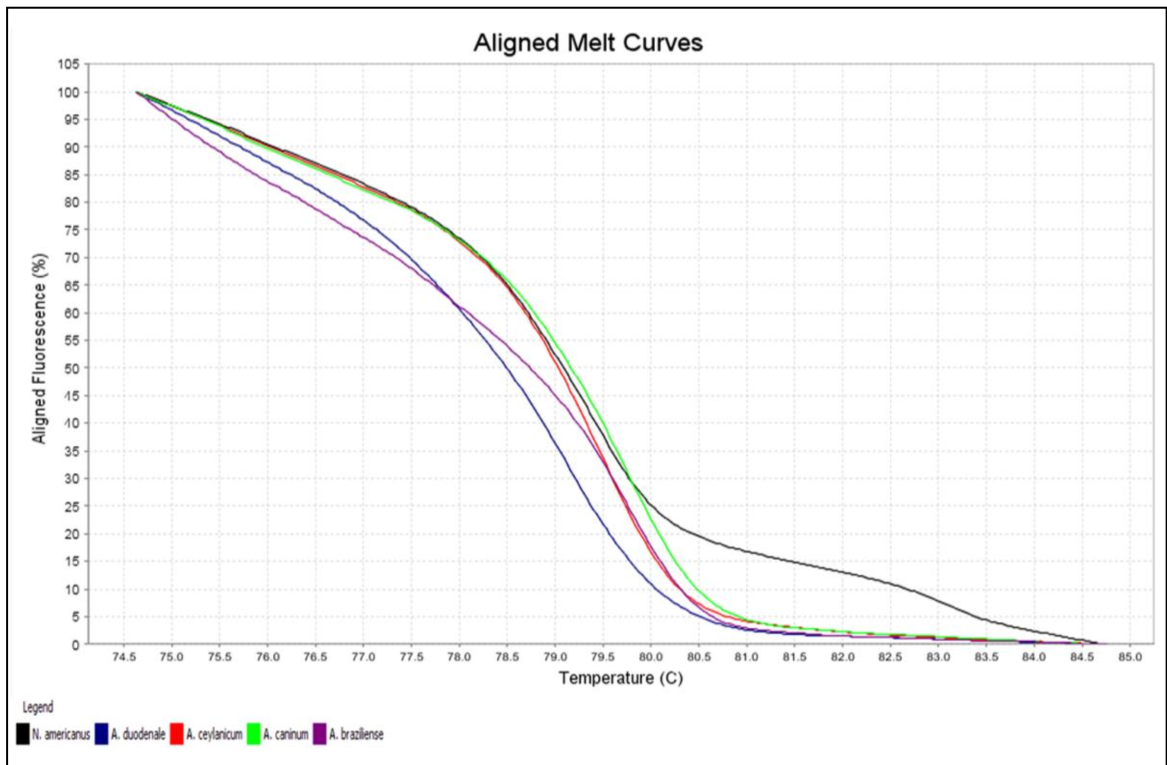

Figure 5.3: Representative profiles of the melting curves (aligned melt curves) of ITS-2 amplicons for *Necator americanus* (black), *Ancylostoma duodenale* (blue), *A. ceylanicum* (red), *A. caninum* (green) and *A. braziliense* (purple). Fluorescence is plotted against degrees Celsius (°C)

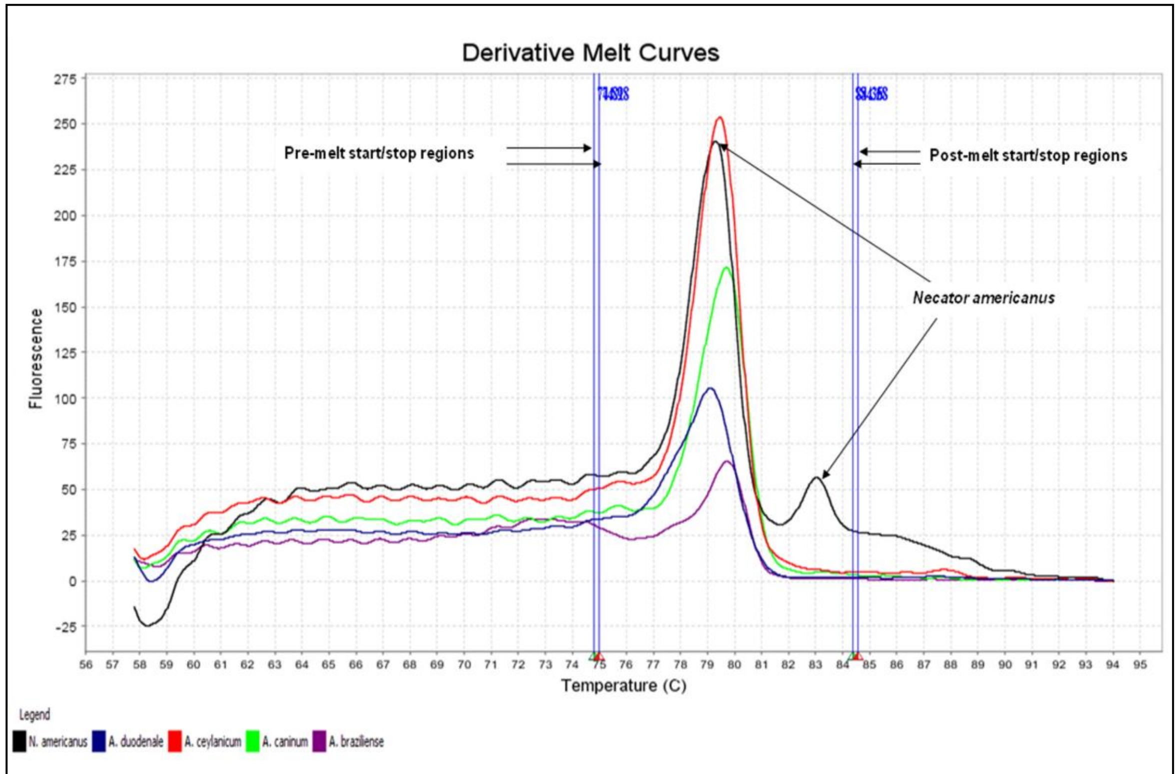

Figure 5.4: Representative profiles of the melting curves (derivative melt curves) of ITS-2 amplicons for *Necator americanus* (black), *Ancylostoma duodenale* (blue), *A. ceylanicum* (red), *A. caninum* (green) and *A. braziliense* (purple). *N. americanus* (black) produced two peaks while single peak was produced for other *Ancylostoma* spp

Pre-melt region: The set of lines to the left of the peak indicates the pre-melt start and stop temperatures when every amplicon is double-stranded

Post-melt region: The set of lines to the right of the peak indicates the post-melt start and stop temperatures when every amplicon is single-stranded

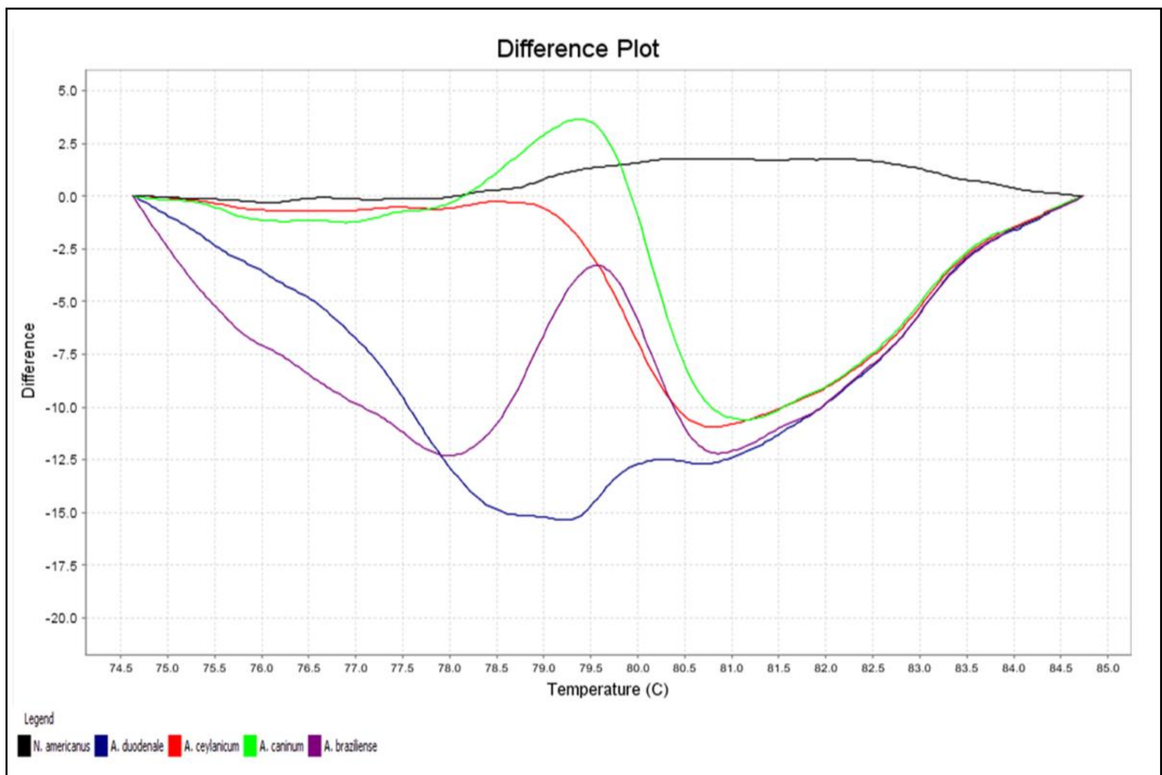

Figure 5.5: Representative profiles of the melting curves (difference plot curves) of ITS-2 amplicons for *Necator americanus* (black), *Ancylostoma duodenale* (blue), *A. ceylanicum* (red), *A. caninum* (green) and *A. braziliense* (purple)

Table 5.1: Results achieved by real-time PCR coupled HRM analysis of ITS-2 amplicon from control genomic DNA for hookworm

Melting curve analysis	**Number of control samples examined**	**Mean melting temperature ± standard deviation (SD)**	
		Peak 1 (T_m1)	**Peak 2 (T_m2)**
Profile 1 (*N. americanus*)	10	79.24 ± 0.05	83.00 ± 0.04
Profile 2 (*A. duodenale*)	10	79.12 ± 0.10	-
Profile 3 (*A. ceylanicum*)	10	79.40 ± 0.10	-
Profile 4 (*A. caninum*)	5	79.63 ± 0.05	-
Profile 5 (*A. braziliense*)	5	79.70 ± 0.14	-

Table 5.2: Hookworm infections detected by microscopy, conventional PCR and real-time PCR-HRM assay of human fecal samples in Peninsular Malaysia (N=634)

Location	**No. Examined**	**Microscopy**		**Conventional Semi-Nested PCR**		**Real-Time PCR-HRM**	
		n	**%**	**n**	**%***	**n**	**%***
Gurney	141	27	19.1	23	16.3	27	19.1
Pos Iskandar	113	14	12.4	11	9.7	14	12.4
Kuala Pangsun	54	6	11.1	6	11.1	6	11.1
Bukit Serok	99	8	8.9	5	5.1	8	8.9
Sungai Bumbun	54	2	3.7	2	3.7	2	3.7
Sungai Layau	89	1	1.1	0	0	1	1.1
Sungai Miak	30	0	0	0	0	0	0
Kemensah	54	0	0	0	0	0	0
Total	**634**	**58**	**9.1**	**47**	**7.4**	**58**	**9.1**

* Based on total number examined (N=634)

Table 5.3: *Necator americanus* and *Ancylostoma ceylanicum* infections in human fecal samples detected by real-time PCR-HRM assay according to villages in Peninsular Malaysia (N=58)

Location	**PCR Positive**	***N. americanus* only**		***A. ceylanicum* only**		***N. americanus* and *A. ceylanicum***	
		n	**%**	**n**	**%**	**n**	**%**
Gurney	27	22	81.5	2	7.4	3	11.1
Pos Iskandar	14	12	85.7	1	7.1	1	7.1
Kuala Pangsun	6	4	66.7	2	33.3	0	0
Bukit Serok	8	7	87.5	0	0	1	12.5
Sungai Bumbun	2	1	50.0	1	50.0	0	0
Sungai Layau	1	1	100	0	0	0	0
Sungai Miak	0	0	0	0	0	0	0
Kemensah	0	0	0	0	0	0	0
Total	**58**	**47**	**81.0**	**6**	**10.3**	**5**	**8.6**

Table 5.4: Comparison between microscopy, conventional semi-nested PCR and real-time PCR-HRM assays (N=634)

Microscopy		**Conventional Semi-Nested PCR [a]**				**Real-Time PCR-HRM [b]**			
		Positive		**Negative**		**Positive**		**Negative**	
	n	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Positive	58	47	81.0	11	19.0	58	100	0	0
Negative	576	0	0	576	100	0	0	576	100

[a] Sensitivity: 84.1%; Specificity: 100% (Figure 5.6)

[b] Sensitivity: 100%; Specificity: 100% (Figure 5.6)

Table 5.5: Hookworm species detected via both conventional semi-nested PCR and real-time PCR-HRM assays (N=58)

Hookworm species	**Conventional Semi-Nested PCR**		**Real-Time PCR-HRM**	
Necator americanus	36	62.1	47	81.0
Ancylostoma ceylanicum	6	10.3	6	10.3
Mixed (*N. americanus and A. ceylanicum*)	5	8.6	5	8.6
Negative	11	19.0	0	0
Total	**58**	**100**	**58**	**100**

1. Sensitivity and specificity of conventional semi-nested PCR

True Positive=58; True Negative =576; False Positive=0; False Negative=11

Sensitivity = True positive ÷ (True positive + False negative) × 100
= 58 ÷ (58 + 11) × 100
= 84.1%

Specificity = True negative ÷ (True negative + False positive) × 100
= 576 ÷ (576 + 0) × 100
= 100%

2. Sensitivity and specificity of HRM-real-time PCR assay

True Positive=58; True Negative =576; False Positive=0; False Negative=0

Sensitivity = True positive ÷ (True positive + False negative) × 100
= 58 ÷ (58 + 0) × 100
= 100%

Specificity = True negative ÷ (True negative + False positive) × 100
= 576 ÷ (576 + 0) × 100
= 100%

Figure 5.6: Calculation of the sensitivity and specificity for both conventional semi-nested PCR and HRM-real-time PCR assay

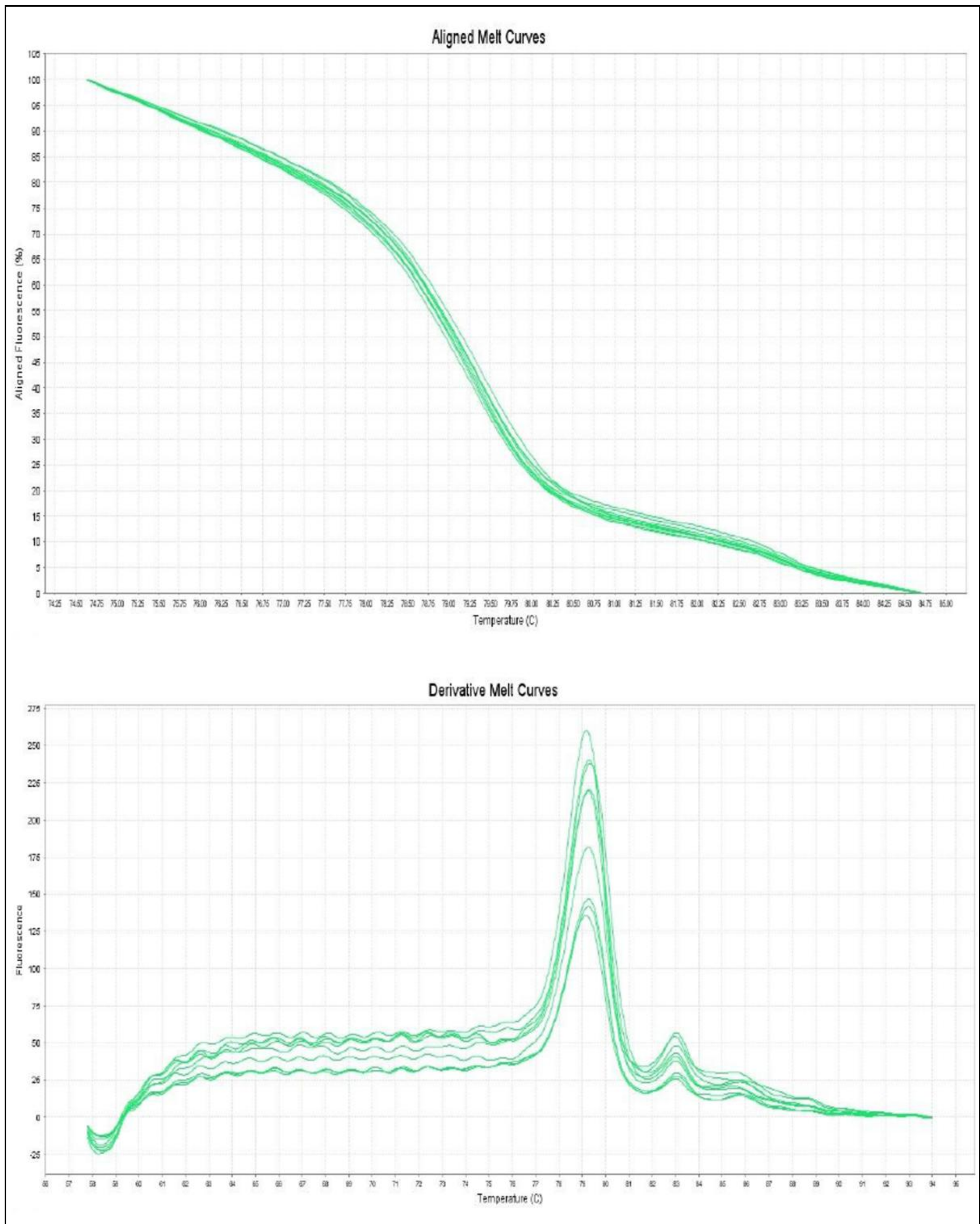

Figure 5.7: The HRM profile (i.e., normalized fluorescence curves) (above) and derivative melt curve (below) of nine out of 11 samples in which hookworm-like eggs were seen via microscopy however failed to be amplified in our conventional PCR. These samples were amplified and identified as *N. americanus* based on their melting profile in HRM assay

5.4 DISCUSSION

It is known that the majority of epidemiology studies of parasitic worm infections, including hookworm species, rely mainly on conventional microscopy as the diagnostic gold standard. Although this method is technically simple and not costly, it is hampered by the fact that most nematode parasites, especially from the Strongyloididae family, are morphologically indistinguishable from each other. Besides that, the technique is laborious to perform, time-consuming and requires skilled personnel. In recent years, several molecular techniques including direct sequencing or probes-based PCR assay have been developed for the specific identification and characterization of hookworm infection (Gasser et al, 1993; 1998; 2006b; Hawdon, 1996; Romstad et al, 1997; Chilton & Gasser, 1999; Traub et al, 2004; de Gruijter et al, 2005a; b; 2006; Verweij et al, 2001; 2007; Basuni et al, 2011). Although these techniques are sensitive and specific for the identification of hookworm species, they are laborious and time consuming, especially the post-PCR processing steps. In addition, there is also a higher risk of contamination, they are more expensive (e.g., DNA sequencing) and the techniques only provide qualitative information.

The present study have successfully utilized HRM analysis coupled with real-time PCR for the rapid detection, quantification and species identification of hookworm species in human samples. HRM analysis is a completely closed-tube system, probe-based genotyping assay that does not employ additional post-PCR steps and simply utilizes a DNA melting assay and computerized analysis to produce graphic output, thus lowing the risk of contamination (Wittwer et al, 2003; Reed & Wittwer, 2004; Montgomery et al, 2007). It measures changes in the rate of double stranded DNA (dsDNA) dissociation to single stranded DNA with increasing temperature. HRM

analysis starts with PCR amplification of the region of interest in the presence of a dsDNA-binding dye (Ririe et al, 1997; Wittwer et al, 2003). This binding dye has high fluorescence when bound to dsDNA and low fluorescence when in the unbound state. When the dsDNA dissociates (melts) into single stranded DNA, the dye is released, causing a change in fluorescence. Amplification is followed by a high-resolution melting step. The observed melting behavior is characteristic of the particular DNA products as determined based on their composition, length, GC content, complementarity and nearest neighbor thermodynamics (Ririe et al, 1997; Wittwer et al, 2003).

To the best of our knowledge, this is the first report on the utilization of the HRM approach for rapid detection and discrimination of nematode (i.e., hookworm) infection by employing the ITS-2 of nuclear ribosomal DNA as a genetic marker. Since the first introduction of HRM analysis, it has been widely applied in clinical studies such as in mutation scanning (Wittwer et al, 2003; Chou et al, 2005; Willmore-Payne et al, 2005; 2006), genotyping of single base changes (Liew et al, 2004), sequence matching (Zhou et al, 2004), insertions or deletions (Radvansky et al, 2010) and detection of single nucleotide polymorphisms (SNPs) (Gundry et al, 2003; Wittwer et al, 2003; Liew et al, 2007; Saitsu et al, 2010). As for parasitic organisms, it has been used sporadically and mainly for the study of parasitic protozoa such as for rapid detection of point mutations associated with antimalarial drug resistance in *Plasmodium falciparum* genes (Mangold et al, 2005; Andriantsoanirina et al, 2009). Similarly, this technique has been applied for the differentiation of Old World *Leishmania* spp. in both human and animal samples (Nicolas et al, 2002; Nasereddin & Jaffe, 2010; Talmi-Frank et al, 2010). Robinson and co-workers (2006) used melting curve analysis of ITS to distinguish the various *Naegleria* species. Similarly, Pangasa and colleagues (Pangasa

et al, 2009) applied the ITS-2 spacer for the rapid screening of several *Cryptosporidium* spp., while the small ribosomal subunit was used as a tool in the HRM analysis of the genetic diversity of different clinical isolates of *Dientamoeba fragilis* (Hussein et al, 2009). Additionally, a similar technique has been used in parasitic worm studies such as for a rapid identification of the two closely related filariasis worms (i.e., *Brugia malayi* and *Brugia pahangi*) (Areekit et al, 2009). More recently, similar method has also been developed for effective population studies of *Fascioloides magna* (Radvansky et al, 2011).

The present study has shown that HRM can be used to easily distinguish among various hookworm species based on the distinctive characteristics of the repeatable curves and melting temperatures for each species although samples were obtained from different hosts (i.e., human vs. animals), sources (i.e., feces vs. adult worm) and life cycle stages (i.e., eggs vs. adult worm). The results revealed that similar melting curves and profiles were generated regardless of whether the sources of the genomic DNA were derived from adult worms or eggs found in human or animal feces. This finding was in accordance with a recent study on the identification of Old World *Leishmania* using a similar approach in which the melting curve was reproducible despite the fact that the *Leishmania* strains originated from different locations, hosts (i.e., human vs. reservoir host) and vectors (i.e., sand flies) (Talmi-Frank et al, 2010).

In this assay, discrimination between the different genera of *N. americanus* and *Ancylostoma* spp. was straightforward as shown by the presence of double-peaks for *N. americanus* but only a single peak for *Ancylostoma* spp. Similar findings were observed in the study of Old World *Leishmania* in which distinctly different curves were produced for non-leishmanial trypanosdomatids and *Leishmania* spp. (Talmi-Frank et al, 2010). Additionally, the appearance of multi-peaks for *N. americanus* was

reproducible and has been demonstrated in various HRM analysis studies (Reed & Wittwer, 2004; Monis et al, 2005; Montgomery et al, 2007; Rasmussen et al, 2007). The presence of multi-peaks can be used as an additional diagnostic criterion for species or genotype discrimination. More recently, a study conducted to differentiate *Cryptosporidium* species using HRM analysis also found multi-peaks for *C. hominis* but not for *C. parvum* or *C. meleagridis* (Pangasa et al, 2009). In general, different genotypes have their own unique transitions that are shown by their HRM profile, shape comparison and difference plots of their melting curve (Wittwer et al, 2003). In the case of double peaks, the lower T_ms peaks were always smaller than the higher peaks, showing the heteroduplex genotype of the melting transition while samples with a single peak indicated a homozygous genotype (Wittwer et al, 2003).

Conventionally, hookworm detection by microscopy is not able to differentiate between *N. americanus* and *Ancylostoma* spp. In the present study, molecular analysis was used to confirm human infections of two species of hookworm, namely *N. americanus* and *A. ceylanicum*. It is assumed that the principal species of hookworm infecting human in Peninsular Malaysia is the most predominant human hookworm worldwide (i.e., *N. americanus*). Thus, it is not surprising that our molecular analysis based on real-time PCR-HRM assay showed that almost all of the infections (89.6%) were caused by *N. americanus*. Similarly, the only available species-specific hookworm study conducted in Sarawak (East Malaysia) also found one *N. americanus* isolate in human sample (Romstad et al, 1998). Interestingly, no *A. duodenale* infection was found in these study areas, which also supports the geographic restriction of this species. This finding was consistent with those of other studies in countries in Southeast Asia, which reported that the distribution of *N. americanus* is far more prevalent than that of *A. duodenale*.

In the southern part of Thailand, a low prevalence for *A. duodenale* (0.1%) has been reported in humans compared with 99.9% for *N. americanus* (Anantaphruti et al, 2002). Recently, a study among persons living in central Thailand indicated that 92.0% of the persons were infected with *N. americanus* compared with 2.0% with *A. duodenale* (Jiraanankul et al, 2011). Conversely, a recent study in Laos found that A*ncylostoma* spp. infections (9.4%) were slightly more prevalent than *N. americanus* infections (5.9%) (Sato et al, 2010). However this finding could be a biased representation because specific species of *Ancylostoma* was not elucidated in that study. As these communities lived in close contact with dogs, it is highly possible that the *Ancylostoma* spp. identified could be a mixture of *A. ceylanicum* and/or *A. duodenale*. Nonetheless, geographic variance in the distribution of the two human hookworm species is a multi-factorial phenomenon, given that factors such as human and parasite behavior, ethnicity, climate, temperature, and environmental factors are involved (Hoagland et al, 1978; Beaver et al, 1984). Given that Malaysia, Thailand, and Laos are neighboring countries with similar geographic conditions, human factors such as life style should be compared in future studies.

Zoonotic ancylostomiasis in humans have been overlooked because of lack of molecular tools to identify hookworm species. In the present study, *A. ceylanicum* infections, a common canine and feline hookworm, were found in human, a finding that in agreement with recent studies in Laos (Sato et al, 2010; Conlan et al, 2012) and Thailand (Traub et al, 2008; Jiraanankul et al, 2011). This result clearly indicated that *A. ceylanicum* may be more common than previously thought and implied that dogs and cats may act as the probable sources of infection to humans. This is particularly relevant in the current study where uncontrolled populations of dogs and cats exist in close proximity with their owners and human often share a close relationship with them. The

possible role of dogs and cats as reservoirs for zoonotic ancylostomiasis in these socioeconomically disadvantaged communities in rural settings where conditions such as poor levels of environmental and sanitary behavior along with a lack of proper veterinary attention and disease awareness that exacerbates the risks of disease transmission will be discussed in **Chapter 6**.

The HRM analysis reported here also revealed that the approach has the capability to detect 'mixed' infections in which uniquely distinct melting curves were produced from previously classified genomic DNA positive with *N. amercianus* and *A. ceylanicum*, a finding that also in agreement with our conventional semi-nested PCR results. A number of previous studies have reported mixed hookworm infections in humans via utilization of conventional methods such as DNA sequencing and RFLP analysis. For instance, a recent study conducted in Thailand revealed that a participant was harboring a mixed infection of *N. americanus* and *A. ceylanicum* as detected by direct DNA sequencing (Traub et al, 2008; Jiraanankul et al, 2011; Conlan et al, 2012). Similarly, mixed infections of *N. americanus* and *A. duodenale* were also reported in Lao PDR (Sato et al, 2010) and Ghana (de Gruijter et al, 2005). This finding is in keeping with a study conducted on the diagnosis of human cryptosporidiosis using a similar HRM analysis, in which the assay could detect mixed infections of *C. hominis* and *C. parvum* prior to SSCP analysis (Pangasa et al, 2009). However, Pangasa and co-workers (2009) also noted that the ability of HRM analysis to detect mixed infection is not expected to achieve consistent sensitivity and accuracy compared to other probe-based genotyping methods such as SSCP. Thus, this limitation can be overcome in future study by combining the current HRM assay with other assays such as multiplex-tandem PCR (Stanley & Szewczuk, 2005) or probe-PCR (Poulson & Wittwer, 2007).

The current HRM assay is more sensitive and specific for the detection and differentiation of hookworm species compared to the conventional semi-nested PCR, as evidenced by the achievement of 100% sensitivity and specificity for the detection of *N. americanus* and *A. ceylanicum* (i.e., previously confirmed based on DNA sequencing data). In addition, 11 samples in which hookworm-like eggs were observed via microscopy but failed to be amplified through conventional semi-nested PCR were identified as *N. americanus* based on the melting profile using our real-time PCR-HRM assay. All microscopy negative samples were also subjected to real-time PCR- HRM assay to make sure that they were not misdiagnosed (i.e., false negative cases of hookworm infection). The results were in accordance with our conventional semi-nested PCR assay where none of the microscopically negative samples were amplified via the HRM assay. Additionally, the ability of the current HRM assay to detect as low as 0.01 ng/µl DNA and its inability to produce any amplification of control DNA representing a wide range of non-hookworm intestinal nematodes and protozoa indicated its potential as an alternative diagnostic tool to other probe-based genotyping assays.

Thus, the current real-time PCR assay coupled with HRM analysis can serve as an alternative molecular epidemiology tool for rapid screening of large numbers of samples. The advantages over other methods used for species differentiation and discrimination include its rapidness, simplicity as no electrophoresis is required to verify the product sustainably reducing processing time, as well as its high specificity and sensitivity. Moreover, HRM analysis does not require a special instrument as it can be performed using an existing real-time PCR system. Likewise, the melting profiles are recorded automatically, stored electronically in a spreadsheet format and can be retrieved at any time point for comparative analyses. Because of its simplicity, HRM analysis offers a cost-effective yet accurate alternative to other probe-based genotyping

assays such as SSCP, RFLP and DNA sequencing. This approach could be applicable to a wide range of microorganisms of medical importance especially closely related species diagnosed in a clinical laboratory.

5.5 CONCLUSIONS

The present study has successfully developed and evaluated the potential use of HRM analysis coupled with real-time PCR assay for the species identification, differentiation and quantification of hookworm infections. It also offers the potential role of this approach for rapid diagnosis and speciation of hookworm infection which can serve as a reliable alternative diagnostic tool to a traditional technique such as microscopy, conventional PCR and probe-based genotyping assays.

The following conclusions are a synopsis of the analysis undertaken through this study in which they were discussed:

1. An evaluation of the method's sensitivity and specificity indicated that this assay was able to detect as low as 0.01 ng/µl hookworm DNA and amplification was only recorded for hookworm positive samples.

2. A unique and distinct melting characteristic of HRM curves (normalized fluorescence curves, derivative melt curve and difference plot melt curve) were produced for each of the five hookworm species which can easily distinguish each species from one another.

3. The melting curves were characterized by peaks of 79.24 ± 0.05°C and 83.00 ± 0.04°C for *N. americanus*, 79.12 ± 0.10°C for *A. duodenale*, 79.40 ± 0.10°C for *A. ceylanicum*, 79.63 ± 0.05°C for *A. caninum* and 79.70 ± 0.14°C for *A. braziliense*.

4. The differentiation between the different genera of *N. americanus* and *Ancylostoma* spp. was straightforward as shown by the presence of double-peaks for *N. americanus* but only single peak for *Ancylostoma* spp.

5. All of the microscopically positive human fecal samples (58/58; 100%) were successfully characterized on the basis of its unique and distinct characteristics of their melting curve patterns using our real-time PCR-HRM assay. However, only 81.0% (47/58) were successfully amplified and genetically characterized using semi-nested conventional PCR on the basis of its DNA sequence.

6. Based on the real-time PCR-HRM assay results, *N. americanus* (52/58; 89.6%) was the most predominant hookworm species detected in human fecal samples, followed by *A. ceylanicum* (11/58; 19.0%). No *A. duodenale* infection was detected in this study. Of this, 81.0% (47/58) and 10.3% (6/58) of humans had single infections with *N. americanus* and *A. ceylanicum*, respectively and 8.6% (5/58) had mixed infections with both species.

7. The specificity evaluation between real-time PCR- HRM assay and conventional semi-nested PCR shown that both assays gave 100% specificity (i.e., the ability of the assay to identify true positive hookworm infections).

8. With regards to the sensitivity (i.e., the ability of the assay to identify true negative hookworm infections), real-time PCR-HRM assay (100%) had higher sensitivity as compared to conventional semi-nested PCR (84.1%).