

CHAPTER 1

GENERAL INTRODUCTION

Soil-transmitted helminth (STH) infections are among the most widespread of human illness especially among those living in areas of poverty in the tropics and subtropics of the developing world. The World Health Organization (WHO) and United States for Disease Control and Prevention (CDC) have recognized STH infections as one of the most neglected tropical disease group together with other diseases including protozoa, bacterial, viral, fungal and ectoparasitic infections (Hotez et al, 2009). Of particular worldwide importance are the two anthropophilic species of hookworm (i.e., *Necator americanus* or *Ancylostoma duodenale*), the roundworm (i.e., *Ascaris lumbricoides*) and the whipworm (i.e., *Trichuris trichiura*) (Chan et al, 1994a). There are considered together because it is common for an individual to be chronically infected with all three worms.

The STH infections are most significant in the bottom billion of the world's poorest people (i.e., income <US\$1.25 per day) (Liese et al, 2010), trapping them in a vicious cycle of poverty and destitution (Hotez et al, 2009). The greatest numbers of infections occur in tropical and subtropical regions of Asia, especially China, India and Southeast Asia as well as sub-Saharan Africa and Latin America (Hotez, 2008a). To date, approximately one third of the world's population (~2 billion people) are infected with at least one species of STH (de Silva et al, 2003; Bethony et al, 2006), resulting in up to 135,000 deaths annually (Anon, 2005). Currently, the global disease burden caused by STH infections is estimated 22.1 million disability-adjusted life years (DALYs) lost to hookworm, 10.5 million to *A. lumbricoides*, 6.4 million to *T. trichiura*, giving a combined total of 39 million life years lost due to STH infections (Hotez et al,

2009). This means that the morbidity of STH infections is as significant as tuberculosis (37.4 million DALYs) and malaria (46.5 million DALYs) (Chan et al, 1994a; Liese et al, 2010).

The life cycle of STH species follow a general pattern with adult parasite worm inhabit the human gastrointestinal tract. For *A. lumbricoides* and hookworm, they live in the small intestine while *T. trichiura* inhabit the human colon. They reproduce sexually and produce eggs, which are then passed from human feces to the external environment for further development and maturation. As adult worms, STH can live for years in the human gastrointestinal tract, causing infections which result in chronic and insidious effects on the host health and nutritional status including iron deficiency anaemia (IDA), protein malnutrition, intellectual retardation, physical impairment, growth stunting, cognitive and educational deficits and hindering economic development (Bethony et al, 2006). The greatest morbidity of STH infections is among children and mother of childbearing age (Albonico et al, 1999; Crompton, 2000; Crompton & Nesheim, 2002; Christian et al, 2004; Anon, 2002; 2005).

Currently, there are three major intervention strategies for controlling STH infections including anthelmintic drug intervention, improved sanitation and health education (Savioli et al, 1992). The periodic deworming with inexpensive anthelmintic drugs (e.g., albendazole, mebendazole, levamisole and/or pyrantel pamoate) stands out as the most cost-effective means to control and reduce the morbidity associated with STH infections (Anon, 2002). Although, globally reports of drug resistance are rare, evidences of emerging resistant do exist throughout the world (Albonico et al, 2004; Bethony et al, 2006). Given the potential of resistance to these anthelmintic drugs, there are significant advances in the development of new approaches for the prevention and control of the STH disease such as hookworm vaccine through the support of the

Human Hookworm Vaccine Initiative (Hotez et al, 2003). Despite these advances, limited attention has been paid to the critically important role that specific diagnosis of infection also play in the control of STH disease.

At present, most research conducted on the epidemiology of STH has relied on the use of conventional microscopy method such as formalin ether sedimentation, Kato-Katz or McMaster techniques for the detection of eggs in the feces based on their typical morphology. However, the utilization of microscopic technique is limited by the fact that most of the nematode eggs are morphologically indistinguishable from those of other species, particularly nematodes belonging to the order Strongylida including hookworm species. To overcome this, fecal cultures of larvae is required to allow eggs to develop and hatch to the third-stage larvae (L3s), which can then be identified microscopically to the genus level (Dunn & Keymer, 1986; Hata et al, 1992). Nonetheless, this method is laborious, time-consuming and requires relatively skilled personnel. Despite this limitation, it is likely this method will remain as gold standard for the diagnosis of STH infections in the near future due to its technical simplicity and low cost (Jozefzoon & Oostburg, 1994). However, it is also vital to recognize that accurate diagnosis to species level and genetic characterization of STHs are crucial key factors contributing to the effective control of these parasites. Thus, there is a necessitate for practical, sensitive and specific diagnostic and analytical tools, particularly those based on polymerase chain reaction (PCR) to be applied to address key epidemiology and population genetic questions to support surveillance, treatment and control program of STH infections (Gasser et al, 2006a).

To date, there has been considerable advancement of molecular diagnostic tools based on polymerase chain reaction (PCR) to accurately determine distribution or population of these nematode worms. Substantial progress has been made on the

development of diagnostic and analytical tools for accurate diagnosis of hookworm species compared to *Ascaris* and *Trichuris* species (Gasser et al, 2006a; Gasser et al, 2009). The extensive progress toward advanced and accurate diagnostic tools for hookworm infection might be due to the growing literature report on human acquired animal hookworm infection through zoonotic transmission such as cutaneous larva migrans (CLM) (i.e., creeping eruptions) caused by *Ancylostoma braziliense* (Chaudhry et al, 1989; Malgor et al, 1996; Bouchaud et al, 2000; Manning et al, 2006; Heukelbach et al, 2007; Feldmeier & Schuster, 2012) and eosinophilic enteritis (EE) caused by *Ancylostoma caninum* (Loukas et al, 1992; Croese et al, 1994a; 1994b; Khoshoo et al, 1995; Bahgat et al, 1999). Hookworm-related CLM cases have also been reported from Malaysian patients (Hanjeet et al, 1988; Robson & Othman, 2008; Hamat et al, 2010; Yap, 2010; 2011) and in tourists who had visited Malaysia (Bouchaud et al, 2000; Lederman et al, 2008).

Likewise, zoonotic ancylostomiasis caused by *Ancylostoma ceylanicum* has also been reported in humans especially in the Asia and Southeast Asia regions (Anten & Zuidema, 1964; Velasquez & Cabrera, 1968; Yoshida et al, 1968; Chowdhury & Schad 1972; Traub et al, 2002; Traub et al, 2008; Sato et al, 2010; Jiraanankul et al, 2011; Conlan et al, 2012). In addition, the migration of zoonotic hookworm larvae can also produce other clinical manifestation in humans including localized myositis, erythema multiforme and ophthalmological symptoms (Bowman et al, 2010). In contrast, zoonotic infection of *Ascaris* and *Trichuris* is uncommon or rarely reported. Previous molecular epidemiology studies conducted in both human and animals host indicated that animal species such as *Ascaris suum* and *Trichuris suis* are not significant source of ascariasis and trichuriasis in human population (Anderson, 1995; Peng et al, 2007; Cutillas et al, 2009; Liu et al, 2012; Ravasi et al, 2012).

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, for example conventional or semi-nested PCR (Gasser et al, 1993; Chilton & Gasser, 1999; Romstad et al, 1997; Verweij et al, 2001; de Gruijter et al, 2005a), PCR-based restriction fragment length polymorphism (RFLP) (Hawdon, 1996; Traub et al, 2004), mutation scanning (Gasser et al, 1998), single-stranded conformation polymorphism (SSCP) analysis (Gasser et al, 2006b), amplified fragment length polymorphism (AFLP) and random amplification of polymorphic DNA (RAPD) (de Gruijter et al, 2005b; 2006) have been developed for the differential diagnosis of hookworm infections in both human and animal host. Likewise, several techniques have been successfully developed to study the genetic variability within and among hookworm species to investigate their epidemiology and population genetics using a marker that show more intraspecific sequence variation such as mitochondrial (mt) genes including cytochrome c oxidase subunit 1 (*cox 1*) and NADH dehydrogenase subunit 1 (*nad 1*) (Zhan et al, 2001, Hawdon et al, 2001; Hu et al, 2002; 2008; Li et al, 2004).

Although these techniques have been successfully utilized for the accurate species discrimination, the post-PCR steps such as gel electrophoresis can be rather time consuming to perform. Likewise, the amplification and detection of DNA were prone to contamination with the endpoint reading on agarose gels yielding no quantitative information. In recent years, the introduction of multiplex real-time PCR using fluorescent TaqMan probes through the possibility of combining assays for the detection of different targets into one reaction has been developed for the specific species identification of hookworm species infecting human (Verweij et al, 2007; Basuni et al, 2011), however consumables especially the labeled-probes are 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 more advanced detection methods which obviate the need for electrophoretic analysis, reduce the risk of contamination and decrease labor time and reagent costs. For example, the introduction of high-resolution melting (HRM) coupled with real-time PCR analysis is a relatively new technique that allows direct characterization of PCR amplicons in a closed-tube system with no manual post-PCR analysis, multiplex steps and relatively low reaction cost (Wittwer et al, 2003; Reed & Wittwer, 2004; Montgomery et al, 2007).

To date, the application of HRM technique in parasitic studies has been rather limited and mainly been used in studies of parasitic protozoa such as *Leishmania* spp. (Nicolas et al, 2002; Nasereddin & Jaffe, 2010; Talmi-Frank et al, 2010), *Cryptosporidium* spp. (Pangasa et al, 2009), *Plasmodium* spp. (Mangold et al, 2005; Andriantsoanirina et al, 2009), *Dientamoeba fragilis* (Hussein et al, 2009), *Naegleria* spp. (Robinson et al, 2006) and *Giardia* spp. (Bienz et al, 2001). As for parasitic helminths, the application of the HRM method has been rather sporadic. Such approach has been used for rapid identification and differentiation of *Brugia malayi* and *Brugia pahangi* (Areekit et al, 2009) and *Fascioloides magna* (Radvansky et al, 2011). By extending such approach on parasitic nematode worms particularly for hookworm infection, it can offers us an alternative tool for the rapid identification and differentiation of hookworm species as a reliable alternative to traditional microscopy, conventional PCR or probe-based genotyping tools.

Besides this advancement and improvement in the diagnostic tools, it is also vital to understand the helminth biology and epidemiology with regards to their environmental and ecological limits. In recent years, there has been renewed interest

from the international organizations in the helminth control program that leads to an increase momentum to attain more comprehensive data, allowing available control resources to be most rational and cost-effectively deployed (Brooker & Michael, 2000). As a result of these changes in health priorities, tremendous efforts have been made in the development of methods to map the distribution of diseases, particularly through the use of geographical information system (GIS) and remote sensing (RS) (Brooker & Michael, 2000). Such approach also made the data integration and mapping more accessible and reliable.

A principal advantage of GIS is that it facilitates regular updating of the database and provides a ready basis for mapping and analysis. It also offers us the ability for modeling the spatial distribution of STH infections in relation to the ecological factors which are derived from remote sensed satellite data that are known to influence their distribution pattern, thus deepening our knowledge and understanding in the biology and epidemiology of the infections (Hay, 2000; Brooker et al, 2006). It allows us to predict the distribution of infection and identify endemic areas, thus providing more precise estimates of populations at risk and map their distribution by facilitating the stratification of areas using infection risk probabilities. This can provide basic information on treatment intervention or public health measure delivery systems at broad spatial scale particularly in areas without comprehensive data (Brooker et al, 2006). In addition, the GIS and RS approach have the potential in facilitating and assisting the design of sustainable development control program at realistic scale for national control program by providing the relevant authorities with relatively low-cost approach for both the upstream (e.g., survey and design) and downstream (e.g., targeting, monitoring and evaluation) control program, which significantly reduce the

cost of practical program by identifying priority areas or simplifying the monitoring and evaluation processes (Brooker et al, 2006).

To date, the GIS and RS tools have been widely used for mapping and modeling of several parasitic diseases including filariasis (Sabesan et al, 2000; Gyapong et al, 2002), malaria (Omumbo et al, 2005; Hay & Snow, 2006; Hay et al, 2006), human African trypanosomiasis (Cecchi et al, 2009), onchocerciasis (Ngoumou et al, 1994; Katabarwa et al, 1999; Noma et al, 2002), schistosomiasis (Malone et al, 2001; Brooker et al, 2002a, 2002b; Kabatereine et al, 2004; Clements et al, 2006; Tchuem Tchuente et al, 2012) and loiasis (Diggle et al, 2007; Zoure et al, 2011).

As for STH infections, GIS and RS approach has been increasingly used for effective storage, mapping and modeling in the development of STH atlas (Brooker & Michael, 2000; Brooker et al, 2000; 2002a; 2002b; 2004, 2009; Knopp et al, 2008; Pullan et al, 2011; Tchuem Tchuente et al, 2012), however such approach in STH mapping has been attempted only in African countries. More recently, the GIS and RS approach for mapping of STH infections has been extended to Southeast Asia regions including Mekong countries (i.e., Cambodia, Lao PDR, Myanmar, Thailand and Vietnam) (Brooker et al, 2003) as well as Indonesia and the Philippines (Brooker, 2002c). By extending such an approach to Malaysia, a reliable and accessible GIS database consists of prevalence map and information of environmental and ecological factors that correlate with their distribution can be explained. It also offers the opportunity to investigate and highlight with greater precision where such information is lacking, identifies and priorities target areas and estimates population at risk and its implications for STH national control program in Malaysia. The establishment of the database for STH infections is imperative for developing and implementing of

sustainable control measures to those populations in greatest need particularly when the recourses for control program are finite and limited.

1.1 Objectives of the study

1.1.1 General objective

The main objective of this study was to develop a novel diagnostic technique and geospatial database for soil-transmitted helminthiasis in Malaysia. However in order to achieve these positive outcomes, initial work in obtaining appropriate samples for HRM and current data for GIS had to be conducted in some epidemiological studies in the human and animal populations. With this in mind, the present study was divided into five chapters (i.e., **Chapter 3 to 7**) representing each specific objectives.

In **chapter 3**, the study involved the screening of human fecal samples in order to obtain positive hookworm samples for the development of a new alternative diagnostic tool using real-time PCR coupled with high resolution melting analysis (HRM), which will be discussed in **Chapter 5**.

Chapter 4 described the prevalence of intestinal helminths in dogs and cats in the communities evaluated in **Chapter 3**. These results will also be used to obtain positive hookworm samples for the development of a new alternative diagnostic tool using real-time PCR coupled with high resolution melting analysis (HRM), which will be discussed in **Chapter 5**.

Chapter 5 encompassed one of our main objectives which was to develop a novel alternative diagnostic tool for rapid detection, quantification and speciation of hookworm infection using real-time PCR coupled with high resolution melting analysis (HRM) targeting the second internal transcribed spacer (ITS-2) ribosomal RNA as genetic marker.

In **Chapter 6**, the study involved the further investigation of zoonotic potential of *A. ceylanicum* isolates recovered from the feces of humans, dogs and cats living in the same communities in rural Peninsular Malaysia using more variable locus (i.e., *cox 1* gene) as complementary to ITS-2 gene.

Finally, **Chapter 7** encompassed one of our main objectives which was to develop a geospatial database of soil-transmitted helminthiasis using geographic information system (GIS) and remote sensing (RS) satellite derived environmental data.

1.1.2 Specific objectives

- 1) To determine the epidemiology of human soil-transmitted helminthiasis among five subgroups of Orang Asli living in remote and semi-remote areas in Peninsular Malaysia (**Chapter 3**).
- 2) To determine the prevalence of intestinal helminthic infections in dogs and cats in these Orang Asli communities (**Chapter 4**).

- 3) To develop a new alternative diagnostic tool for rapid detection, quantification and speciation of hookworm using real-time PCR coupled with high resolution melting (HRM) analysis targeting the second internal transcribed spacer (ITS-2) ribosomal RNA as a genetic marker (Chapter 5).
- 4) To further investigate the zoonotic potential of *A. ceylanicum* isolates recovered from the feces of humans, dogs and cats living in the same communities in rural Peninsular Malaysia (Chapter 6).
- 5) To develop a geospatial database for soil-transmitted helminthiasis using the geographic information system (GIS) and remote sensing (RS) derived environmental data (Chapter 7).

1.2 Research hypotheses

Objective 1 (i.e., **Chapter 3**): To determine the epidemiology of human soil-transmitted helminthiasis among five subgroups of Orang Asli living in remote and semi-remote areas in Peninsular Malaysia.

- 1) The socioeconomic, environmental sanitation and personal hygiene characteristics are generally poor among the survey population.
- 2) The prevalence of all three STH infections (i.e., *T. trichiura*, *A. lumbricoides* and hookworm) are high among the surveyed populations.

- 3) The distribution patterns of STH infections are significantly different among different Orang Asli subgroups.
- 4) The prevalence of STH infections are significantly higher among vulnerable group such as children compared to adults.
- 5) The prevalence of STH infections is significantly higher among those with low socioeconomic background, poor sanitation, environmental and personnel hygiene among the surveyed populations.

Objective 2 (i.e., **Chapter 4**): To determine the prevalence of intestinal helminthic infections in dogs and cats in these Orang Asli communities.

- 1) The prevalence of intestinal helminth infections is expected to be high in dogs and cats.
- 2) Dogs and cats are definitive hosts for several intestinal helminth species which have zoonotic potential.

Objective 3 (i.e., **Chapter 5**): To develop a new alternative diagnostic tool for the rapid detection, quantification and speciation of hookworm using real-time PCR coupled with high resolution melting (HRM) analysis targeting the second internal transcribed spacer (ITS-2) ribosomal RNA as genetic marker.

1. A new alternative diagnostic tool using real-time PCR coupled with high resolution melting analysis (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 specific of this assay is expected to be improved compared to microscopy and conventional semi-nested PCR.

Objective 4 (i.e., **Chapter 6**): To further investigate the zoonotic potential of *A. ceylanicum* isolates recovered from the feces of humans, dogs and cats living in the same communities in rural Peninsular Malaysia.

1. The common species of hookworm in dogs and cats include *A. ceylanicum*, *A. caninum* and *A. braziliense*.
2. There will be a considerable level of genetic variation within the *cox 1* sequences of *A. ceylanicum* isolated from humans, dogs and cats.

3. Some of the *A. ceylanicum* strains from the same geographical location and host are expected to be clustered together within the same group.
4. There will be high number of nucleotide substitution (e.g., transversions and transitions) at different locations within the *Ancylostoma* genus. These mismatches of nucleotide will allow differentiation between *Ancylostoma* species.

Objective 5 (i.e., **Chapter 7**): To develop a geospatial database of soil-transmitted helminthiasis using the geographic information system (GIS) and remote sensing (RS) derived environmental data.

1. The number of individual with combined infection of any STH species is estimated accurately using a simple probabilistic model.
2. The prevalence map showed the geographical distribution of STH infections from any available survey data and highlighting where such data is lacking.
3. The spatial distribution of STH infections is influenced by the environmental factors.
4. The predictive risk map of STH infections in areas without comprehensive data is generated.

5. The total number of population and school-aged children who are at risk of infection and warranting anthelmintic treatment is estimated.

1.3 Significance of the study

The establishments of molecular tools combined with conventional diagnostic and epidemiological tools for hookworm infection will allow the accurate determination of parasite prevalence, increase the understanding of transmission dynamics and provide important information on the zoonotic potential of hookworm in high risk community. In these socioeconomically disadvantaged communities, the poor levels of hygiene and overcrowding, lack of veterinary attention and zoonotic awareness along with uncontrolled population of dogs or cats that exist in close proximity with humans, exacerbate the risks of zoonotic transmission in these communities. There is a crucial need for a simple, rapid and highly sensitive and specific technique such as the method reported in this study that can serve as an alternative analytical tool to improve diagnosis and surveillance of hookworm infections. In addition, the complementary use of a more variable locus such as the *cox 1* gene as complementary to the ITS-2 gene will provide additional evidence to support the zoonotic exchange of hookworm species particularly *A. ceylanicum* between humans and animals.

With regards to the utilization of geographic information system (GIS) and remote sensing (RS), it can play important roles and provide basic information for the implementation of sustainable and effective control programs for STH infections in Malaysia. For instance, although many surveys have been conducted particularly in endemic areas in Malaysia, an accurate estimation of the total disease burden has not been fully explained. Additionally, these data are rarely in the public domain and

available in a form that is accessible to policy makers or relevant authorities for control program intervention. The use of GIS allows us to collate and map the geographical distribution of STH infections from available empirical survey data in Malaysia. It also offers the opportunity to investigate the geographical distribution of infection highlighting where such information is lacking. Such approach has been further enhanced by the used of RS tool that provides proxy to the environmental factors and correlate with the prevalence data in order to identify ecological correlation with infection patterns. The correlations between infection patterns and ecological factors can be used to extrapolate predictive risk map in areas for which no data are available. Likewise, the predictive risk map can serve as a baseline data to estimate number of population at risk, numbers of requiring treatment and cost of delivering anthelmintic. Establishment of such reliable map is essential for the development and implementation of control measures to those populations in greatest need particularly when the recourses for control program are finite and limited. Therefore, findings of the current study will be valuable for the public health authorities to justify and facilitate the reassessment of the existing control measures to reduce the prevalence of STH infections.