INTESTINAL PARASITES AND HOOKWORM SPECIES IN STRAY CATS, DOGS AND SOIL: AN EPIDEMIOLOGICAL APPROACH TO STUDY SOIL CONTAMINATION WITH ZOONOTIC PARASITES

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF MEDICAL SCIENCE

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2016

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Registration/Matric No: MGN130037

Name of Degree: Master of Medical Science

Title of Project Paper/Research Report/Dissertation/Thesis (~~"t~~his Work"):

INTESTINAL PARASITES AND HOOKWORM SPECIES IN STRAY CATS, DOGS AND SOIL: AN EPIDEMIOLOGICAL APPROACH TO STUDY SOIL CONTAMINATION WITH ZOONOTIC PARASITES

Field of Study: Parasitology

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ABSTRACT

The study was conducted to determine the prevalence of intestinal helminth eggs and protozoa excreted in the faeces of stray cats and dogs as well as in soil samples. A total of 379 fresh faecal samples (from 227 dogs and 152 cats) and 126 soil samples were collected. The egg and (oo)cyst stages were detected via microscopy after the application of formalin–ether concentration technique. Genomic DNA was extracted from the samples containing hookworm eggs and used for further identification to the species level using real-time polymerase chain reaction coupled with high resolution melting analysis. Microscopic observation showed that the overall prevalence of helminth eggs among stray cats and dogs was 75.7% (95% CI = 71.2%–79.9%), of which 87.7% of dogs and 57.9% of cats were infected with at least one parasite genus. Five genera of helminth eggs were detected in the faecal samples, including hookworms (46.4%), *Toxocara* (11.1%), *Trichuris* (8.4%), *Spirometra* (7.39%) and *Ascaris* (2.37%). The prevalence of helminth infections among stray dogs was significantly higher than that among stray cats ($p < 0.001$). Only three genera of helminths were detected in soil samples with the prevalence of 23% (95% CI = 15.1%–31%), consisting of hookworms (16.6%), *Ascaris* (4%) and *Toxocara* (2.4%). The molecular identification of hookworm species revealed that *Ancylostoma ceylanicum* was dominant in both faecal and soil samples. The dog hookworm, *A. caninum*, was also detected among cats, which is the first such occurrence reported in Malaysia to date. This finding indicated that there was a cross-infection of *A. caninum* between stray cats and dogs because of their coexistence within human communities. As for protozoa (oo)cysts, the overall prevalence was 20.3%, with 22.4% in cats and 18.9% in dogs. Four genera of protozoa (oo)cysts were detected, including *Giardia* (8.2%), *Isospora* (3.4%), *Cyclospora* (4.2%) and *Cryptosporidium* (4.5%). Only two genera of protozoa were detected in soil samples with the prevalence of 9.5% in which *Isospora* (7.1%)

being the commonest protozoan detected, followed by *Giardia* (2.4%). Taken together, these data suggest the potential role of stray cats and dogs as being the main sources of environmental contamination with zoonotic intestinal parasites which potentially involve in human infections.

ABSTRAK

Kajian ini telah dijalankan untuk menentukan prevalens helmin dan protozoa usus dalam tinja kucing dan anjing terbiar dan juga dalam sampel tanah. Sejumlah 379 sampel tinja segar (dari 227 anjing dan 152 kucing) dan 126 sampel tanah telah dikutip. Peringkat telur dan (oo)sista telah dikesan melalui mikroskopi setelah diaplikasikan teknik kepekatan formalin-eter. DNA genomic telah diekstrasikan dari sampel yang mengandungi telur cacing kait dan seterusnya digunakan untuk identifikasi ke peringkat spesies menggunakan teknik tindak balas polimerase berantai _real-time' bersama dengan analisis _high resolution melting'. Pemeriksaan mikroskopi menunjukkan prevalens keseluruhan telur helmin dikalangan kucing dan anjing terbiar adalah 75.7% (95% CI = 71.2% - 79.9%), dimana 87.7% anjing dan 57.9% kucing telah dijangkiti dengan sekurang-kurang satu genus parasit. Lima genera telur helmin telah dikesan dalam sampel tinja, termasuk cacing kait (46.4%), *Toxocara* (11.1%), *Trichuris* (8.4%), *Spirometra* (7.39%) dan *Ascaris* (2.37%). Prevalens jangkitan helmin dikalangan anjing terbiar adalah lebih tinggi secara signifikant dari kalangan kucing terbiar ($p < 0.001$). Hanya tiga genera helmin telah dikesan dalam sampel tanah dengan prevalens 23% (95% CI = 15.1% - 31%), terdiri dari cacing kait (16.6%), *Ascaris* (4%) dan *Toxocara* (2.4%). Pengenalpastian molekul bagi spesies cacing kait menunjukkan yang *Ancylostoma caninum* adalah dominan dalam kedua-dua sampel tinja dan tanah. Cacing kait anjing, *A. caninum*, juga telah dikesan dikalangan kucing, dimana yang pertama dilaporkan kewujudannya di Malaysia. Penemuan ini menunjukkan terdapat jangkitan silang *A. caninum* diantara kucing dan anjing terbiar oleh sebab kewujudan bersama mereka dalam komuniti manusia. Bagi (oo)sista protozoa, prevalens keseluruhan adalah 20.3%, dengan 22.4% dalam kucing dan 18.9% dalam anjing. Empat genera (oo)sista protozoa telah dikesan, termasuk *Giardia* (8.2%), *Isospora* (3.4%), *Cyclospora* (4.2%)

dan *Cryptosporidium* (4.5%). Hanya dua genera protozoa telah dikesan dalam sampel tanah dengan prevalens 9.5% dan *Isospora* adalah protozoa yang paling lazim dikesan (7.1%) diikuti oleh *Giardia* (2.4%). Data ini mengesyorkan potensi peranan kucing dan anjing terbiar sebagai punca utama mengkontaminasi alam sekitar dengan parasit (usus) zoonotik yang berpotensi melibatkan jangkitan dalam manusia.

ACKNOWLEDGEMENTS

First of all with great humility, I dedicatedly accord my sincere gratitude to Almighty God for His benign blessing hand and bestowing a creative and healthy environment throughout this work.

My eternal thanks to my supervisors, Associate Professor Dr. Init Ithoi and Professor Dr. Rohela Mahmud, for providing me supervision, ideas, assistance and advice in every step throughout this project. Their endless help and generous information have transformed this research project into a priceless scientific output.

I am also grateful to Dr. Awatif Mohamed Abdulsalam Salih (Department of Parasitology, FOMUM) and Mr. Boon Pin (Department of Molecular Medicine, FOMUM) for their advice and technical assistance in the laboratory. I would also like to thank Dr. Romano Ngui for providing me hookworm genomic DNA for positive controls during my molecular works. My appreciation also goes to all the academic and non-academic staffs of Department of Parasitology who were involved either directly or indirectly in the completion of this project. Thanks are also due to my friend, Ms Izyan Samsudin for her help and assistance in the field trips. Special thanks also go to animal shelters‘ personnels for their assistance during faecal samples collection.

Finally, I‘d like to thank my beloved parents, family members and all my friends for giving me moral support, encouragement and believing in me throughout this project.

THANK YOU EVERYONE

Sandee Tun

May 2016

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

et al.	:	et alia (others)
spp.	:	species
g	:	gram
%	:	percent
eg	:	example
ml	:	milliliter
mm	:	millimeter
IPIs	:	intestinal parasitic infections
PCR	:	polymerase chain reaction
DALYs	:	disability-adjusted life years
i.e.	:	id est
µm	:	micrometer
°C	:	degree Celsius
Etc	:	et cetera
IDA	:	iron deficiency anaemia
DNA	:	deoxyribonucleic acid
SSCP	:	single-strand conformation polymorphism
RFLP	:	restriction fragment length polymorphism
DPX	:	distyrene plasticizer xylene
HCl	:	hydrochloric acid
µl	:	microliter
HRM	:	high resolution melting
pmole	:	picomole
ng	:	nanogram

GI	:	gastrointestinal
SPSS	:	statistical package for the social sciences
CI	:	confidence interval
OR	:	odd ratio
X^2	:	Pearson‘s chi-square

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Intestinal parasitic infections (IPIs) are global health burden that occur in tropical and subtropical regions worldwide, particularly endemic in developing countries (Apidechkul, 2015). It is estimated that more than three billion people are infected with intestinal parasites (helminths and protozoa) in the world (Balcioglu *et al*., 2007). Intestinal parasites, including soil-transmitted helminths (STH) (e.g. *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms) and protozoa (e.g. *Giardia duodenalis*, *Isospora* spp. and *Cryptosporidium* spp.) are indeed regarded as serious public health problems, causing clinical morbidity of 450 million people especially among children and women of reproductive age (Al-Mohammed *et al*., 2010; Alaofè *et al*., 2008; Quihui *et al*., 2006). The prevalence and distribution of IPIs always depend on several factors such as personal hygiene, dietary habits, education level of the community and climate conditions. Moreover, social-economic status, sanitary and environmental conditions also determine the underlying causes of endemicity of parasitic infections in the communities (Al-Mohammed *et al*., 2010; Balcioglu *et al*., 2007). However, the greatest risk of transmission appears to be from companion animals such as cats and dogs, as they are susceptible to and excellent carriers of many zoonotic parasites.

Cats and dogs are vulnerable hosts to many zoonotic intestinal parasites that can be transmitted through direct contact or through faecal materials. Since the abundant population of cats and dogs and their roles as hosts for parasites of intestinal zoonotic diseases towards human infections, studies of zoonotic parasites associated with cats and dogs have been carried out worldwide (Wiwanitkit & Wiwanitkit, 2015; Millan & Casanova, 2009; Ngui *et al*., 2014; Traub *et al*., 2014; Palmer *et al*., 2007). In Malaysia, intestinal parasites among cats and dogs are noted from several genera such as

Toxocara, *Ascaris*, *Trichuris*, hookworm, *Taenia*, *Spirometra*, *Strongyloides*, *Giardia*, *Cryptosporidium* and *Isospora* (Ngui *et al*., 2014; Mahdy *et al*., 2012; Azian *et al*., 2008).

The concern of the public health has increased considerably as the potential of zoonotic transmission from gastrointestinal parasites of cats and dogs have been reported previously (Macpherson, 2013; Traub, 2013; Wiwanitkit & Wiwanitkit, 2015; Schär *et al*., 2014; Ngui *et al*., 2013; Palmer *et al*., 2010). Zoonosis occurs when there is a close intimacy with animals either as pets or when they are freely roaming around defaecating and contaminating the human environment with fertile eggs and infective (oo)cysts in the faecal materials. Such behavioral characteristics of stray cats and dogs with human environment, including defecating and scavenging rubbish, can easily lead to the contamination of soil within their roaming territories. Moreover, these stray animals are not regularly subjected to anti-parasitic treatment and contribute to maintenance of parasitic life cycles in the environment (Waap *et al*., 2014).

Soil pollution with faecal materials is instrumental in the transmission of parasites mainly soil-transmitted helminths (STHs) infections. The examination of the soil for STH parasites performed in Thailand revealed that *Ascaris* spp., *Trichuris* spp., *Toxocara* spp. and hookworm species are the main parasites that could be transmitted by means of contaminated soil (Waenlor & Wiwanitkit, 2007). The fertilised eggs of STH (*A. lumbricoides, Ancylostoma* spp., *Toxocara* spp., *T. vulpis, Toxascaris leonina,* etc.) deposited in the soil develop rapidly and may reach an infective stage within a matter of weeks depending on environmental conditions. Because infected stray animals shed eggs around public places, healthy animals and humans may acquire infections due to the contaminated environments. Therefore, the zoonotic parasites are linked to soil contamination by free-roaming stray animals, which are sources of human

infections. Children are often at risk of zoonotic infections due to their affections for pets, geophagia and the playing habits such as playing with the soil that has already been contaminated with infected animals‘ faeces.

STH infections have been categorized as neglected tropical diseases (NTDs) as these infections are most common among the poorest people living in the developing countries causing tremendous disability and suffering (Hotez *et al*., 2008). These infections give significant burden to the billions of the world‘s poorest people (i.e daily income < US $2) (Bethony *et al*., 2006). The disease burden caused by these infections is accessed by disability-adjusted life years (DALYs) (Mathers *et al*., 2007) causing global disease burden estimated to be approximately 22.1 million DALYs lost to hookworm infections, 10.5 million to ascariasis and 6.4 million to trichuriasis (Hotez & Kamath, 2009).

Many studies have reported STH infections as the leading cause of malnutrition, mental and growth retardation, cognitive impairment and educational implication particularly in school-aged children (Kitvatana & Rhongbutsri, 2013; Crompton & Nesheim, 2002; Alaofè et al., 2008; Bethony *et al.*, 2006). The consequences of STH infections such as acute *Ascaris* intestinal obstruction, hepatobiliary and pancreatic ascariasis (HPA) (Crompton, 2001), *Trichuris* dysentery syndrome (TDS) or rectal prolapse with persistent bloody diarrhoea (Brooker & Bundy, 2014), anaemia and iron deficiency anaemia (IDA) (Stoltzfus *et al*., 1997) are often reported in young children. Moreover, the adverse effects of hookworm infections among pregnant women include neonatal prematurity, abortion, increased maternal morbidity and mortality (Brooker *et al*., 2008). Other manifestations, such as human eosinophilic enteritis and cutaneous larva migrans or creeping eruptions, have been found to be caused by zoonotic hookworms (Landmann & Prociv, 2003; Bowman *et al*., 2010; Costa *et al*., 2008;).

The prevalence of zoonotic protozoan infections was low, yet it still contributes to the significant burden of gastrointestinal illness around the world (McHardy *et al*., 2014). The impact of these infections was often reported in the developing world where inadequate sanitation, poor hygiene and proximity to zoonotic reservoirs, particularly companion animals are common (Thompson & Smith, 2011). Intestinal zoonotic protozoa such as *Giardia duodenalis*, and opportunistic protozoa such as *Cryptosporidium* spp. and *Isospora* spp. are recognised as food and water born protozoa and the transmission is mainly through faecal-oral routes via contaminated food and water in which stray cats and dogs are responsible for contamination of the environments.

Following 2013 sporadic outbreaks in the United States, the pathogenicity of *Cryptosporidium* spp., *Isospora* spp. and *Cyclospora* spp. have been unveiled (McHardy *et al*., 2014; Fletcher *et al*., 2012). These protozoan species have been identified as the main causes of diarrhoea especially in children and immunocompromised patients (Al-Mohammed *et al*., 2010; Norhayati *et al*., 2003). They are also reported among the travellers coming back from the endemic areas and described as travelers‘ diarrhoea (Goodgame, 2003). It is noted that approximately 4 billion diarrhoeal cases occur globally each year which account for 4% of all deaths (Torgerson & Macpherson, 2011) and 59 million DALYs lost per year (Lopez *et al*., 2006). In addition, many studies have reported *Giardia* and *Cryptosporidium* as zoonotic enteric parasites, especially in cats and dogs, and the occurrence is of potential significance from clinical and public health perspectives (Hunter & Thompson, 2005; Uehlinger *et al*., 2013; Ballweber *et al*., 2010; Thompson *et al*., 2008;).

A number of studies have been carried out worldwide to determine the prevalence of intestinal parasites among stray cats and dogs, and the prevalence varies

from as low as 11.9% to as high as 94% (Tudor, 2015; Overgaauw & Nederland, 1997; Waap *et al*., 2014; Palmer *et al*., 2008; 2007; Sommerfelt *et al*., 2006; Oliveira-Sequeira *et al*., 2002). This major variation in prevalence may be due to the geographical distribution of intestinal parasites, and the variation in the natural environment which provides the survival of these parasites. In Malaysia, little information is known on the prevalence of gastrointestinal parasites among stray cats and dogs. Zain *et al.* (2013) reported that 74.6% of stray cats in Malaysia were infected with helminths. In addition, they reported hookworm species as being the most prevalent, including *A. braziliense* (30.8%) and *A. ceylanicum* (31.5%). This finding was further supported by Ngui *et al.* (2014) that helminth infections were significantly more common than those of protozoa among cats and dogs of Malaysia and hookworms, *Ancylostoma* species, were the most prevalent (71.4%).

Hookworms are blood-feeding parasites that inhabit the intestine of mammalian hosts, including cats, dogs and humans. The most common hookworms in cats are *A. braziliense* and *A. ceylanicum*, whereas those in dogs are *A. caninum* and *A. ceylanicum*. In humans, two main species are prevalent, namely *Necator americanus* and *Ancylostoma duodenale* (Chan *et al*., 1994), coupled with zoonotic hookworms (Inpankaew *et al*., 2014; Ngui *et al*., 2012c; Khoshoo *et al*., 1995). The clinical manifestations of hookworm infections in humans include epigastric pain, diarrhoea and iron-deficiency anaemia, all of which can lead to malnutrition as well as mental and growth retardation, particularly in children (Hsu & Lin, 2012; Bethony *et al*., 2006; Brooker *et al*., 1999; Bahgat *et al*., 1998; Olsen *et al*., 1998; Stoltzfus *et al*., 1997). Other manifestations, such as human eosinophilic enteritis (*A. caninum*) and cutaneous larva migrans or creeping eruptions (*A. braziliense*), have been found to be predominantly caused by specific species of zoonotic hookworms (Landmann & Prociv, 2003; Prociv & Croese, 1996; Bowman *et al*., 2010; Costa *et al*., 2008).

Diagnosis of the helminth eggs by microscopic observation has been used for many decades (Ritchie, 1948); however, molecular methods have achieved the best results for the identification of worm species. Several molecular studies have been carried out over the past few years to identify hookworm species, including conventional and semi-nested polymerase chain reaction (PCR) (Ngui *et al*., 2012b), single-strand conformation polymorphism (SSCP) (Hu *et al*., 2002), mutation scanning (Gasser *et al*., 1998) and PCR–restriction fragment length polymorphism (RFLP) (Traub *et al*., 2004). However, these methods are time consuming, with higher risk of contamination during the molecular processes, and relatively expensive. Hence, considering all these factors, real-time PCR with high resolution melting analysis was used for rapid detection and screening of hookworm species in the present study.

1.2 Statement of research problem

In Malaysia, a large population of stray cats and dogs are seen roaming within human communities. Some people sympathize with them, offer them food and allow them to freely roam in human environment. Little do people aware that, these stray animals are also hosts to many zoonotic parasites and close contact with them can expose humans to zoonotic diseases. Many studies have been done worldwide to investigate the association between these animals and zoonotic infections among humans (Wiwanitkit & Wiwanitkit, 2015; McCarthy & Moore, 2000; Ngui *et al*., 2014; Palmer *et al*., 2010). Moreover, stray animals are also environmental contaminators as they contaminate the soil when they defaecate faeces together with infective helminth eggs or protozoa (oo)cysts in the human environment (Tudor, 2015; Tun *et al*., 2015).

Although there are increasing concerns about the burden of these stray animals towards public health, little information is known on the burden of zoonotic diseases

from stray animals. Therefore, it would be useful to detect the existence of possible zoonotic parasites among these stray animals and soil samples contaminated with their faeces. Collection of faecal samples among these stray cats and dogs as well as soil samples from different areas can assist in obtaining the estimate of parasitic infections to the population of stray cats, dogs and the status of soil contamination with intestinal parasites from these stray animals.

1.3 Objectives of the study

1.3.1 General objective

The general objective of this study was to determine the current distribution of zoonotic parasites in stray cats, dogs and soil in Klang Valley, Malaysia.

1.3.2 Specific objectives

1. To determine the current prevalence of intestinal parasites (zoonotic) among stray cats and dogs in Klang Valley, Malaysia.
2. To determine the occurrence of intestinal parasites (zoonotic and anthroponotic) in soil of randomly chosen public places and recreational parks of Klang Valley, Malaysia.
3. To identify the hookworms into species levels found among stray cats and dogs as well as soil samples in study areas.

1.4 Research hypotheses

1. The prevalence of intestinal helminths is higher than those of protozoa among stray cats and dogs in Klang Valley, Malaysia.
2. The prevalence of soil-transmitted helminths (*Ascaris*, *Trichuris*, *Toxocara* and hookworms) is generally high in soil samples randomly selected from the public areas.
3. *Ancylostoma ceylanicum*, is the most common hookworm species found among stray cats, dogs and soil samples in Klang Valley, Malaysia.

1.5 Significance of the study

This study is significant in establishing comprehensive current status of zoonotic parasites among stray cats, dogs and from environmental soil samples. In addition, the current study focuses on identification of hookworms, the most prevalent zoonotic parasites among stray cats, dogs and environment in Malaysia, into species level. The establishment of such data is beneficial for public health services to devise effective control strategies and to raise awareness in local communities.

CHAPTER 2: LITERATURE REVIEW

2.1 Epidemiology of zoonotic intestinal parasites

Cats and dogs are present in every community, every country and every part of the world. They are the hosts to many helminths and protozoan endoparasites, among which have zoonotic potential, are responsible for many gastrointestinal illness and increase concerns in public health (Macpherson, 2013; Traub, 2013; Wiwanitkit & Wiwanitkit, 2015; Schär *et al*., 2014; Ngui *et al*., 2013; Palmer *et al*., 2010;). The prevalence of intestinal parasites among cats and dogs have been reported with prevalence rates varying from 11.9% - 94%, worldwide (Khalafalla, 2011; Millán & Casanova, 2009; Hajipour *et al*., 2015; Lefkaditis *et al*., 2014; Waap *et al*., 2014; Abu-Madi *et al*., 2010; Inparnkaew *et al*., 2007; Sommerfelt *et al*., 2006; Labarthe *et al*., 2004; Calvete *et al*., 1998; Baker *et al*., 1989; Engbaek *et al*., 1984; Nichol *et al*., 1981). Among these intestinal parasites, the prevalent rates for helminths were reported as follows: hookworm species (1.4% - 91%), *Toxocara cati* (0.8% - 79%), *Joyeuxiella pasqualei* (2.5% - 76%), *Diplopylidium acanthotetra* (20.7% - 60%), *Dipylidium caninum* (0.1% - 58%), *Taenia taeniformis* (3.1% - 60%) and *Toxascaris leonina* (0.2 – 30%). As for protozoan parasites, the common species were *Isospora* spp. (1% - 22%), *Giardia* spp. (0.7% - 28.47%), *Cryptosporidium* spp. (1.5% - 55%), *Sarcocystis* spp. (1%- 1.2%), and *Toxoplasma gondii* (9% - 85%) (Khalafalla, 2011; Barutzki & Schaper, 2011; Millán & Casanova, 2009; Waap *et al*., 2014; Ngrenngarmlert *et al*., 2012; Mircean *et al*., 2010; Inpankaew *et al*., 2007; am re -Barrios *et al*., 2004).

In Europe, the intestinal parasites that are commonly reported in exotic canine and feline were hookworm species, *Toxocara* spp., *Giardia* spp., *Cryptosporidium* spp. In Western Europe, the prevalence rates of *T. canis* among dogs varied from 3.5% - 34% whereas for cats, *T. cati* were 8% - 76% (Deplazes *et al*., 2011; Martínez-Moreno *et al*., 2007; Overgaauw, 1997). The parasitic infections were found high among the

younger animals (dogs and cats of age between 3-6 months) from Hungary (Capári *et al*., 2013), Spain (Martínez-Moreno *et al*., 2007), Germany (Barutzki & Schaper, 2011) and Greece (Lefkaditis *et al*., 2014). Moreover, several studies have claimed that the dogs‘ fur was an important source for transmission of toxocariasis in humans (Wolfe & Wright, 2003; Wael *et al*., 2011; Roddie *et al*., 2008). In addition, *T. cati* was significantly higher among female stray cats in Greece (Lefkaditis *et al*., 2014) and the *Toxocara* eggs were also found in soil samples from the park (Mizgajska, 2001; Oge & Oge, 2000; Roddie *et al*., 2008).

As for protozoa, the prevalence of *Giardia* spp. among dogs was reported high in several European countries including Belgium (28.47%), Germany (23.75%), Spain (25.10%), France (27.53%), Italy (25.89%), and United Kingdom (24.62%) (Epe *et al*., 2010). Meanwhile, the prevalence in cat was reported from Belgium (26.31%), Germany (24.59%), Spain (14.59%), France (15.31%), Italy (17.71%), Netherlands (13.20%) and United Kingdom (11.54%) (Barutzki & Schaper, 2011). The *Cryptosporidium* spp. was also found to be common among dogs in western countries such as in Spain (7.4%) (Causape *et al*., 1996), Italy (1.1–3.3%) (Zanzani *et al*., 2014; Rinaldi *et al*., 2008), in kennel dogs of Poland (13%) (Bajer *et al*., 2011), shepherd and hunting dogs of Greece (2.8%) (Papazahariadou *et al*., 2007), owned dogs of the Netherlands (8.7%) (Overgaauw *et al*., 2009) and dogs in rural areas of the Czech Republic (2%) (Dubná *et al*., 2007). Among cats, *Cryptosporidium* spp. was reported from the Netherlands (4.6% in household cats) (Overgaauw *et al*., 2009) and United Kingdom (8.1% - 17.2%) (Scorza *et al*., 2014). In addition, *Cryptosporidium* spp. was also found in wild carnivores, the red foxes from Slovakia (38.7%) (Ravaszova *et al*., 2012) and wolves in Poland (37.5–55%) (Paziewska *et al*., 2007; Kloch *et al*., 2005).

In Southeast Asia (SEA) (according to the United Nations definition, comprises of 11 countries namely; Brunei, Cambodia, East Timor, Indonesia, Lao PDR, Malaysia,

Myanmar, Philippines, Singapore, Thailand and Vietnam), approximately 40%, 36% and 26% of the 563 million people are infected with *Ascaris lumbricoides (*227 million), *Trichuris trichiura* (200 million) and hookworms (149 million), respectively (Utzinger *et al*., 2010; Bethony *et al*., 2006; Brooker *et al*., 2006; De Silva *et al*., 2003). As for zoonotic parasites, hookworms and *Toxocara* species are the major important parasites in the aspect of public health problems (Macpherson, 2013). *Toxocara* is a common roundworm of cats and dogs reported abundantly in countries including Vietnam (Le *et al*., 2016), Malaysia (Ngui *et al*., 2014; Zain *et al*., 2013), Thailand (Inpankaew *et al*., 2007; Jittapalapong *et al*., 2007a), and China (Li *et al*., 2006). In addition, *T. malaysiensis* that are found in cats, may have potential of causing infection in humans especially in the countries with a high endemicity for this species (Le *et al*., 2016). As for protozoa, *Toxoplasma gondii* (tissue protozoa) is the most important parasite, confirmed to have zoonotic potential from mainly cats to humans (Wiwanitkit & Wiwanitkit, 2015). It was reported to be widespread among stray cats in Bangkok metropolitan areas (Jittapalapong *et al*., 2007b) and population control of stray cats as reduction for transmission of toxoplasmosis was suggested (Wiwanitkit & Wiwanitkit, 2015; Jittapalapong *et al*., 2010). Many epidemiological studies were also reported on the zoonotic potential of *Giardia* spp. from cats and dogs (Hunter & Thompson, 2005; Bouzid *et al*., 2015; Ballweber *et al*., 2010). Kitvatanachai and Rhongbutsri (2013) reported that *G. lamblia* was detected in 50% of 202 school children from suburban government schools in Thailand.

In Malaysia, *Ancylostoma* spp. and *Toxocara* spp. were noted to be the major zoonotic parasites. *T. cati* was found in high prevalence (42.9%) in cats while *T. canis* (28.6%) in dogs (Babjee, 1978; Mohd Zain & Sahimin, 2010; Ngui *et al*., 2014; 2013; Zain *et al*., 2013; Ngui *et al*., 2012b; Azian *et al*., 2008; Choo *et al*., 2000; Macadam *et*

al., 1984; Shanta *et al*., 1980). In 2001, a new species, *T. malaysiensis*, was described in Malaysia (Gibbons *et al*., 2001) which previously was catergorized as *T.canis*.

2.2 Parasites contaminating the environmental soil

Cats and dogs either as pets or strays tend to wander outdoors, eat contaminated food they find and are exposed to many parasites including soil transmitted helminths (STHs) such as *Ascaris*, *Trichuris*, *Toxocara*, and hookworm species infections. They also harbour many parasites that are capable of causing diseases to humans. In public places where animals (cats and dogs) are allowed, it has been reported that they defaecate, liberating STH eggs leading to contamination of soil and generating risk to public health (Abou-El-Naga, 2015; Mizgajska, 2001; Morgan *et al*., 2013; Conlan *et al*., 2012; Azian *et al*., 2008; Sommerfelt *et al*., 2006; Giacometti *et al*., 2000). The anthroponotic parasites such as *A. lumbricoides* and *T. trichiura* may also contaminate the environmental soil, via infected faeces directly or indirectly from domestic animals.

A. lumbricoides infection, ascariasis, affects 1.2 billion people around the world, mostly from developing countries where sanitation and hygiene is poor (De Silva *et al*., 2003). It is estimated that ascariasis contributes 10.5 million disability-adjusted life years (DALYs) and morbidity with serious health consequences observed in 122 million cases per year (De Silva *et al*., 2003).

Ascariasis is usually asymptomatic. The clinical features can be classified as acute and chronic manifestations, respectively associated with larval migration and adult worms in the gastrointestinal tract (Bethony *et al*., 2006). Ectopic migration includes the worms wandering into the pancreatic, biliary ducts and the appendix leading to corresponding illnesses. Hepatobiliary and pancreatic ascariasis (HPA) occur when adult worms in the duodenum enter the bile duct, leading to biliary colic,

acalculous cholecystitis, cholangitis, pancreatic and hepatic abscess. HPA is commonly observed among adults (especially females) as the biliary tree is large enough to accommodate the adult worm (Misra & Dwivedi, 2000; Khuroo *et al*., 1990). Gallbladder ascariasis, another significant, yet uncommon endemic pathology of *A. lumbricoides*, often missed out by ultrasonography especially if the worms are dead or the gallbladder contains stones leading to fatal complications such as gallbladder empyema, septicaemia and pericholecystic abscess were also reported (Javid *et al*., 1999). The larvae that die during migration through liver can induce eosinophilic granulomas (Kaplan *et al*., 2001). The migration of the larvae through the lungs can cause respiratory distresses such as wheezing, dyspnoea, fever, and coughing. Heavy infection of lungs may result in verminous pneumonia (Loeffler's syndrome) and children are more susceptible.

The worms inhibiting the intestine can cause a variety of non-specific symptoms such as abdominal pain, discomfort, vomiting, nausea, general restlessness and insomnia. The presence of a large number of worms in the intestine is often associated with lactose intolerance, malabsorption of vitamin A and other essential nutrients leading to reduced physical fitness and growth retardation especially among children (Crompton & Nesheim, 2002; Taren *et al*., 1987). In very young children, high intensity of adult worms can cause mechanical obstruction of the intestine followed by secondary bacterial infections resulting in fatal peritonitis (Bethony *et al*., 2006).

In immunosuppressed individuals, *A. lumbricoides* antigen can act as parasitic allergens leading to hypersensitivity and exhibiting allergic reaction in the lungs, skin, conjunctiva and in the gastrointestinal tract (Brooker & Bundy, 2014). The antigen reaction prod uces high IgE, which is a feature of allergic responses and enhances the likelihood of asthma (Alcântara-Neves *et al*., 2010; Hagel *et al*., 2007; Hunninghake *et al*., 2007; Palmer *et al*., 2002).

T. trichiura (whipworms) affects approximately 800 million people worldwide and the estimated DALY's trichuriasis lost was 6.4 million (Chan, 1997; Bethony *et al*., 2006; De Silva *et al*., 2003). The transmission is mostly due to the accidental ingestion of infective eggs from the contaminated hand, food, soil and water. Although it is found throughout the world, commonly in tropical areas (with heavy rainfall, constant warm temperature and poor sanitation), the highest prevalence occurs in Central Africa, Southern India and Southeast Asia. The adult worm lives in the caecum, but is also found in the appendix and lower ileum (Bethony *et al*., 2006). Crohn's disease and ulcerative colitis occurred due to the inflammation at the attachment sites by large number of worms, leading to bloody mucus diarrhoea, chronic malnutrition, short stature, anaemia, finger clubbing in children (Bundy & Cooper, 1989). Only heavily infected patients (especially children) develop clinical diseases such as dysentery and chronic colitis. However, the significant manifestation of whipworm is *Trichuris* dysentery syndrome (TDS) which is severe diarrhoea with blood and mucus (Bundy & Cooper, 1989). Children with such symptoms are often emaciated and may develop anaemia. Due to the extensive mucosal swelling of the rectum, the infected patients acquire an urge to bear down as if stool is present (tenesmus). Protracted tenesmus can cause rectal prolapse (Bundy & Cooper, 1989). Iron deficiency and growth stunting were reported, which sometimes can be reversible with specific antihelminth drugs (Cooper, 1995; Cooper & Bundy, 1988; Cooper *et al*., 1986).

2.3 Role of cats and dogs to humans and domestic environment

The relationship between humans and cats began about 9500 years ago whereas the archaeological records showed that mankind's best friends, the domestic dogs was likely to date back to the stone age of 12,000-15,000 years ago (Macpherson, 2005; Morey, 1994). Millions of people around the world keep them as pets in the homes and

the closeness exists until this modern world. This human-animal bond is mutually beneficial and provides significant impact in human life (Dennis, 2013; Zasloff, 1996; Gehrt *et al*., 2010; Garrity *et al*., 1989). Cats were reported to be the most popular pets in the world (Driscoll *et al*., 2009). It was estimated that the global population of cats ranges from 200 to 600 million (Gehrt *et al*., 2010) and approximately 30% of the households have cats as pets worldwide (Coleman *et al*., 1997). The domestic cat, *Felis catus,* originated from the ancestral species *Felis silvestris*, the European and African wild cats. Cats are normally considered as loving, elegant, stubborn, quicker to react, independent, quiet, clean and less expensive to pet owners. Some people also want to keep cats in their houses to control pests. Pet cat's lifespan usually is between 15 to 17 years, however, as for stray cats, the survival rate is reduced and estimated to live between 4 to 5 years of age (Ogan & Jurek, 1997). The reproductive maturity age is between 7 to 12 months (Ogan & Jurek, 1997) and normally the female cats can produce two litters per year with an average litter of four kittens (Nowak & Paradiso, 1983). Figure 2.1 shows the map of top ten countries with most pet cat population in the world.

Dogs are considered as obedient, faithful and protective companion of humans. They are domesticated descendants of wolves and it is estimated that there are 500 million dogs around the world (Macpherson, 2005). From both observational and traditional aspects, dogs, the man's best friends, serve as ideal model of animal companionship as they have the ability to pertain human-dog interactions such as taking walk, travelling together, hunting and playing outdoor games (Zasloff, 1996). Dogs can also serve additional role as protectors or deterrents against intruders. With proper training, dogs can also assist humans in many invaluable roles, including working as sheep or cattle dogs, guide dogs for the blind, as sniffer dogs used for detection of drugs, tracker dogs, mountain, sea and avalanche rescue animals as well as police dogs

(Waltner-Toews, 1993; Giles-Corti & Donovan, 2003;). Figure 2.2 shows the top ten countries with most pet dog populations.

Cats and dogs play important roles in societies throughout the world particularly contributing to the physical, social and emotional development of children and the well-being of owners (especially elderly people) (Jennings, 1997; Waltner-Toews, 1993; Zasloff & Kidd, 1994; Robertson *et al*., 1990; 2000). Headey and Krause (1999) reported that pet owners tend to visit their doctor less, use fewer medication and have lower blood pressure and cholesterol levels than non-pet owners (Robertson *et al*., 2000). Studies have found positive association between cardiovascular health and pet ownership including, reduced risk of heart diseases (Anderson *et al*., 1992) and an increased survival rate following a heart attack (Friedmann *et al*., 1980). In addition, reduced feeling of depression, loneliness and increased sense of life satisfaction were reported among senior people (Turner, 2000; Karsh & Turner, 1988; Mahalski *et al*., 1988) and single woman with pets (Zasloff & Kidd, 1994).

Although pets offer significant benefits to our society, there are well-documented health hazards associated with owning a pet (Robertson & Thompson, 2002). Cats and dogs are associated with many zoonotic organisms, in which many enteric pathogens are of particular concern (Ngui *et al*., 2014; Ramos *et al*., 2013; McGlade *et al*., 2003; Hill *et al*., 2000). Schantz (1994) reported the potential role of cats and dogs as reservoirs for zoonotic enteric parasites and the potential health risk towards humans remain a significant problem worldwide.

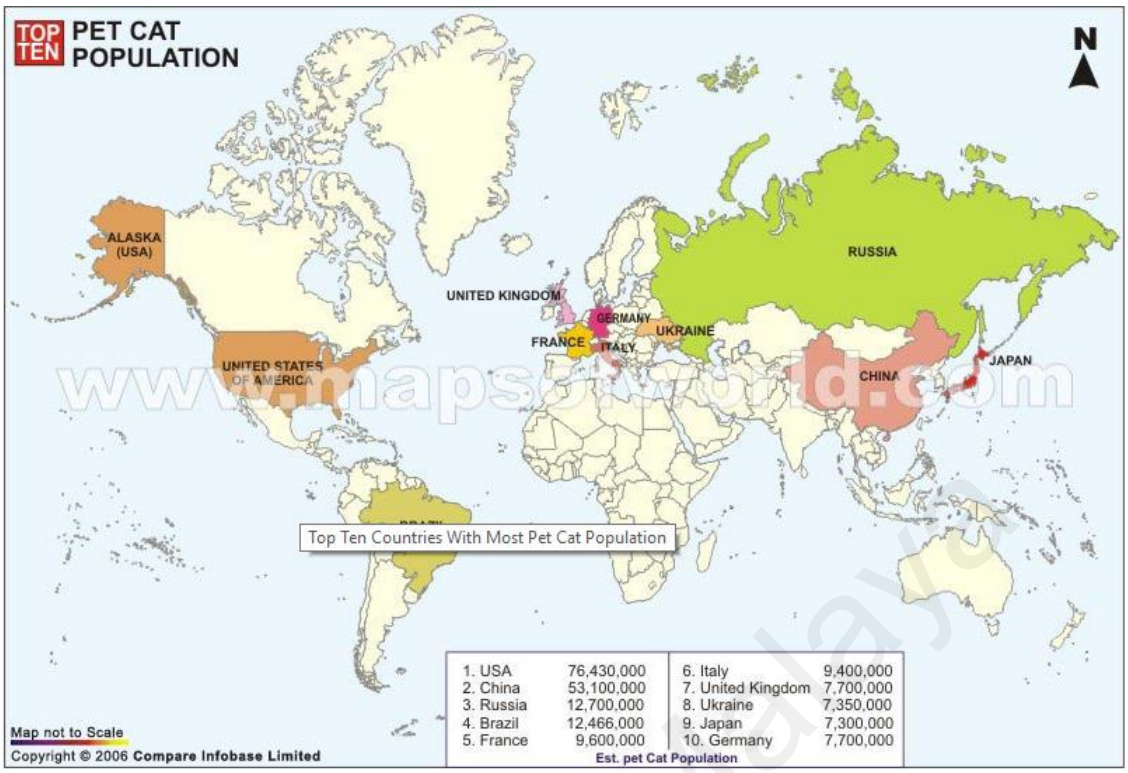

Figure 2.1: Top ten countries with most pet cat population. Source: Maps of World (http://www.mapsofworld.com/world-top-ten/countries-with-most-pet-cat-population.html)

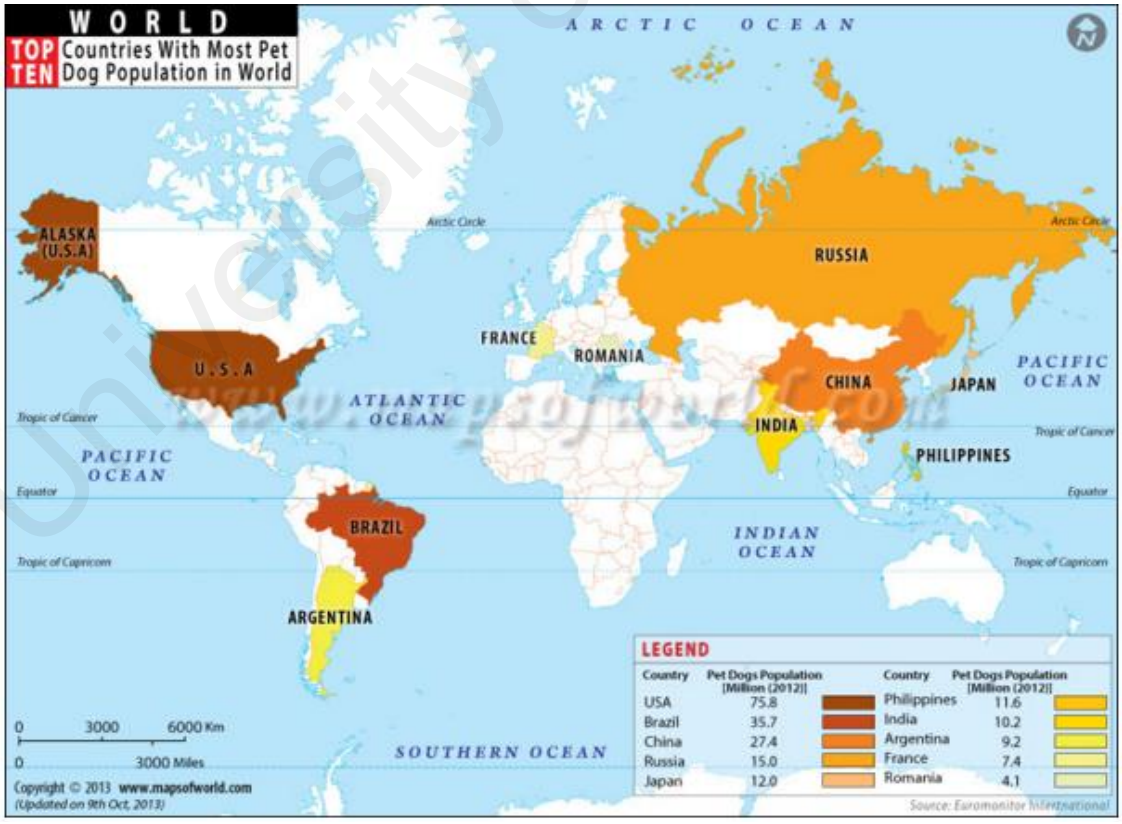

Figure 2.2: Top ten countries with most pet dog population. Source: Maps of World (http://www.mapsofworld.com/world-top-ten/countries-with-most-pet-dog-population.html)

2.4 Common enteric parasites infecting cats and dogs

Cats and dogs, both domestic and stray, have the common characteristics of wandering around the communities in search for food and are exposed to parasitic infections due to the accidental ingestion of contaminated food/water and in some cases, through intermediate hosts such as cockroaches, frogs, fish, rodents etc. In Malaysia, a list of enteric parasites reported among cats and dogs is shown in Table 2.1 (Ngui *et al*., 2014; Zain *et al*., 2013; Mahdy *et al*., 2012; Ngui *et al*., 2012b; Azian *et al*., 2008; Gibbons *et al*., 2001).

Table 2.1: Enteric parasites detected in cat and dog populations in Malaysia

Nematodes	**Protozoa**
[a]*Ancylostoma ceylanicum*	[a]*Giardia* spp.
[a]*Ancylostoma caninum*	[a]*Cryptosporidium* spp.
[a]*Ancylostoma braziliense*	*Eimeria* spp.
[a]*Toxocara cati*	*Entamoeba* spp.
[a]*Toxocara canis*	*Balantidium coli*
[a]*Toxocara malaysiensis*	*Isospora* spp.
[b]*Ascaris* spp.	
[b]*Trichuris vulpis*	
Strongyloides spp.	
Toxascaris leonine	
Physaloptera praeputialis	
Cestodes	**Trematodes**
[a]*Dipylidium caninum*	*Platynosomum fastosum*
Taenia taeniaeformis	
Hymenolepis diminuta	
[a]*Spirometra* spp.	

[a] Zoonotic potential [b]Enviornmental contaminator

2.5 Zoonotic potential of intestinal parasites of cats and dogs in humans

Although numerous intestinal parasites have been recognised in dogs and cats (Table 2.1), not all have the zoonotic potential for transmission to humans (Robertson & Thompson, 2002). The zoonotic parasites that may have high possibility to infect and

causes zoonotic diseases in Malaysian communities are *Giardia*, *Cryptosporidium, Toxocara*, *Spirometra* and hookworm species (Kavana *et al*., 2014; Ngui *et al*., 2014; Zain *et al*., 2013; Ngui *et al*., 2012a; 2012b; Lim *et al*., 2011; Azian *et al*., 2008; Menon *et al*., 2001).

Giardia duodenalis (previously known as *G. lamblia*) is a zoonotic protozoan parasite that infects a wide variety of wild and domestic animals as well as humans worldwide (Uehlinger *et al*., 2013). It is a ubiquitously distributed flagellated protozoan that inhabits the small intestine of its host, where it attaches to the surface of intestinal mucosa. Dogs and cats serve as hosts for *Giardia* species (Bouzid *et al*., 2015; Claerebout *et al*., 2009) and the infection is transmitted through faecal contamination especially from drinking water. Humans have been reported to be infected with *G. duodenalis* and recent molecular data has defined eight genetic assemblages of *G. duodenalis*, A-H. Assemblages A and B were reported in humans, C and D primarily in dogs and assemblage F in cats (Ballweber *et al*., 2010). In addition, assemblages A and B have been isolated from dogs, providing their potential to act as a source of infection (Traub *et al*., 2005). In Malaysia, the reported prevalence range of *Giardia* among cats and dogs was 10.7% - 13% (Ngui *et al*., 2014). In addition, 5.7% of HIV/AIDS patients in Malaysia were reported to be positive with this opportunistic *Giardia* species (Lim *et al*., 2011).

Giardia is endemic in many regions of the world especially the tropics and is more common in children than in adults. It exists in two forms: the trophozoite and the cyst. The former is pear-shaped and motile, has a concave ventral surface which allows it to attach itself to the gut epithelium. There are four pairs of flagella arising from eight basal granules and is binucleated. The infective cyst is oval with four nuclei, has a rigid outer wall that protects it from the harsh environmental conditions such as extreme

temperature, dehydration and disinfectants. Giardiasis is protracted diarrhoea which can be mild and produce semisolid stools or intensive with watery and voluminous stools. If left untreated, the infection can last for weeks to months (Despommier *et al*., 2012). Giardiasis has major impact on children causing insufficient nutrition and growth stunting. The protozoa can restrict the absorptive area of the gut epithelium and cause malabsorption syndrome which gives rise to abdominal pain, headache, nausea, vomiting, malaise and cramping illness (Piekarski, 1989; Kucik *et al*., 2004).

Cryptosporidium is a coccidian protozoan found in the small intestine of many vertebrates, including humans (Fayer, 2010). It has also been detected in dogs and cats (Milstein, 1995; Johnston, 1993; Sargent *et al*., 1998) which represent an important reservoir of infection for humans (Robertson & Thompson, 2002; Robertson *et al*., 2000). The first cryptosporidiosis outbreak in human was reported in 1976 and numerous cases have been reported worldwide since then (Ungar, 1990). It was found in many animals including sheep, horses, cattle, birds, rodents, dogs, cats, etc (Hunter & Thompson, 2005; Uehlinger *et al*., 2013; Paoletti *et al*., 2011; Savioli *et al*., 2006; Olson *et al*., 1997). It is an opportunistic parasite especially among immunodeficient patients, patients suffering from AIDS, infants and young children (Latif & Rossle, 2015; Snelling *et al*., 2007). The infection occurs mostly via faecal-oral route through contaminated food and drinking water (O'Donoghue, 1995; Smith & Grimason, 2003; Savioli *et al*., 2006). Transmission also can occur through person to person either direct or indirect contact, from animals to animals, and from animals to humans (Cieloszyk *et al*., 2012; Fayer *et al*., 2000).

Although 12 valid species of *Cryptosporidium* were reported in mammals, *C. parvum* remains the most important species infecting mammals, particularly humans (Tzipori & Ward, 2002; Fayer *et al*., 2000). Menon *et al*. (2001) reported that 0.9% of

children in Malaysia hospitalized with acute diarrhoea were due to *C. parvum* infections. Moreover, Ngui *et al*. (2014) reported that 7.1% of cats and 6.5% of dogs were infected with *Cryptosporidium* in rural areas of Selangor and Pahang States in Malaysia. The symptoms of cryptosporidiosis vary from mild diarrhoea to watery diarrhoea with loose stools (Stensvold *et al*., 2015). Upper abdominal cramping, nausea and vomiting resulting in hypersecretion of intestinal fluid, loss of water and electrolytes have been reported (Kurniawan *et al*., 2009). The disease is self-limiting, lasting several days in healthy individuals. As for immunnodeficient hosts, the disease is severe, causing loss of approximately 3 liters of fluid per day (Sanad & Al-Malki, 2007), and is always associated with malnutrition or superinfections with other pathogens, leading to death (Sanad & Al-Malki, 2007; Fayer *et al*., 2000).

Toxocariasis is caused by the zoonotic roundworm of dogs (*Toxocara canis*) and/or cats (*Toxocara cati*) (Overgaauw & Nederland, 1997). Both of these species are ascarid nematodes in the order *Ascaridida*, superfamily *Ascaridiodea*, family *Toxocaridae* (Despommier, 2003). They have ubiquitous distribution throughout the world and are common environmental contaminators of human habitats (Mizgajska, 2001; Giacometti *et al*., 2000). The infection in human is mainly due to poor hygiene or accidental ingestion of infective eggs (Brooker & Bundy, 2014). Children frequently have clinical symptoms of toxocariasis due to the playing habits with contaminated soil in the sandpits and yards or geophagia (eating dirt) (Macpherson, 2005; Overgaauw & Nederland, 1997). Dogs and cats serve as important reservoirs for the transmission of diseases to humans (Lee *et al*., 2010). The infections are more common in the tropics and subtropic areas (Despommier, 2003). Other new *Toxocara* species such as *T. malaysiensis*, infecting domestic cats (Le *et al*., 2016; Li *et al*., 2006; Gibbons *et al*., 2001) and *T. lyncus*, infecting the caracal (wild cats) (Macchioni, 1999) have also been reported.

The clinical symptoms of toxocariasis in human are related to the organs affected due to larval migration of the *Toxocara* species. The three main clinical syndromes associated to human infections are ocular larval migrans (OLM), visceral larval migrans (VLM) and convert toxocariasis. The OLM occurs due to the larval migration to the host eyes and/or around the optic nerve and causes ocular toxocariasis (OT). The symptoms of OT include red eyes, leukocoria (white eye), strabismus, retinal granuloma or detachment, pars planitis and finally leading to vision loss or blindness (Lee *et al*., 2010; Smith *et al*., 2009). The VLM is mainly predominant among young children (<5 years old) and comprises of clinical features associated with abdominal pain, fever, anorexia, headache, vomiting, coughing/wheezing, hepatomegaly and weight loss. As for covert toxocariasis, it is a mild form of VLM and include symptoms such as coughing, abdominal pain, headache, sleeplessness and behavior disturbance (Despommier, 2003; Fisher, 2003; Despommier *et al*., 2012; Smith *et al*., 2009). However, most cases of toxocariasis in humans are reported as asymptomatic (Oréfice *et al*., 2016; Hayashi *et al*., 2005).

Sparganosis is a rare zoonotic cestode (tapeworm) disease caused by the plerocercoid or sparganum (third stage larva) of diphyllobothroid tapeworms belonging to the genus *Spirometra* (Liu *et al*., 2015; Li *et al*., 2011). *Spirometra* needs three hosts to complete its lifecycle which are cats and dogs which serve as the important final hosts, cyclops which serve as first intermediate hosts (infected with procercoids), and frogs which are the main second intermediate hosts (infected with plerocercoids (spargana)) (Li *et al*., 2011). Humans acquire infections due to the unusual eating habits of raw or undercooked intermediate hosts such as frogs, cats, dogs, fish and/or through drinking untreated water containing infected copepods with infective larva (Sabu *et al*., 2014; Li *et al*., 2011). Sparganosis has been reported worldwide and is commonly found in East and South East Asian countries including China, India, South Korea, Thailand,

Japan mainly due to the unusual eating habits (Sabu *et al*., 2014; Duggal *et al*., 2011; Nithiuthai *et al*., 2004). Sporadic cases have been reported in South America, Europe, and Africa, with several cases described among travelers returning from endemic regions and dogs and cats are regarded as the most important definitive animal hosts in these endemic regions (Liu *et al*., 2015). Ngui *et al*. (2014) reported that 10.4% of dogs in Malaysia were infected with *Spirometra* species. Moreover, Kavana *et al*. (2014) successfully illustrated the experimental infection of hamster as second intermediate host with *Spirometra* in his lifecycle study research on *Spirometra* species of Malaysia. The sparganum can also invades subcutaneous connective tissues and superficial muscles, manifesting as subcutaneous sparganosis occurs as skin sore or nodule due to the localization of the larva. It is the most common type of sparganosis found, especially in China, Thailand, Japan, and Korea (Liu *et al*., 2015; Kim *et al*., 2014; Anantaphruti *et al*., 2011; Li *et al*., 2011).

Ocular sparganosis occurs when the larvae invade the conjunctiva and the orbit of the eye (Liu *et al*., 2015). When the larvae reach the posterior pole of the eye, they cause periorbital oedema, eyeball protrusion and movement disorder resulting in pain in the eye, excessive lacrimation, ptosis, irritation, orbital cellulitis with marked swelling of the eyelids, and ulceration of the cornea (Liu *et al*., 2015; Ye *et al*., 2012; Anantaphruti *et al*., 2011). When the larvae migrate to the anterior chamber, hypopyon, synechia, and secondary glaucoma (Liu *et al*., 2015; Rehák *et al*., 2006) can cause excessive lacrimation, redness of the eyes, irritation itchiness and pain in the eyes and if untreated, can lead to blindness (Liu *et al*., 2015; Ye *et al*., 2012; Anantaphruti *et al*., 2011; Mentz *et al*., 2011;).

Visceral sparganosis is caused by the migration and growth of the sparganum in the intestine, breast, abdominal cavity, lung and heart, resulting in damage or

malfunction of the particular organ (Koo *et al*., 2011; Lee *et al*., 2011; Iwatani *et al*., 2006) such as intestinal obstruction (or peritonitis) leading to abdominal pain, in the case of intestinal perforation (Liu *et al*., 2015). In rare cases, the larva can also migrate to the spinal canal or cerebral hemispheres and causes cerebrospinal sparganosis. Migration in the brain causes neurological symptoms such as fatigue, limb weakness, confusion, headache, seizure, memory loss, coma, fever, paresthesias, hemiparesis, brain abscesses, and numbness or tingling of skin (Hong *et al*., 2013). Other signs include lower limbs numbness, urinary and bowel incontinence (Park *et al*., 2011).

2.6 Hookworm Species

Hookworm infection was documented to be the highest prevalent infection in cats and dogs in many countries including Malaysia. In addition, zoonotic hookworms diseases in humans continue to be a major health problem in Southeast Asia (Inpankaew *et al*., 2014; Ngui *et al*., 2012a; Jittapalapong *et al*., 2007a). Zoonotic hookworms of dogs were recognized as *Ancylostoma caninum*, *A. braziliense*, *A. ceylanicum* and *Uncinaria stenocephala* while those of cats were *Ancylostoma tubaeforme*, *A. braziliense*, *A. ceylanicum* and *U. stenocephala* (Prociv, 1998; Landmann & Prociv, 2003). Among these, *A. ceylanicum* was the most common in Southeast Asia in the past 45 years (Conlan *et al*., 2011; Sato *et al*., 2010; Traub *et al*., 2008). In Malaysia, *Ancylostoma* species was also reported to be high in both cats and dogs, of which *A. ceylanicum* (31.5-90.6%) and *A. braziliense* (9.4%-30.8%) were detected in stray cats (Zain *et al*., 2013; Yoshida *et al*., 1973). *A. ceylanicum* was also predominant in stray dogs from Kuala Lumpur (Rahman *et al*., 1985) and Sarawak (Choo *et al*., 2000). Hence, widespread of *A. ceylanicum* was noted in humans living in close association with dogs and highlighted the zoonotic transmission of hookworm between dogs and

humans (Ngui *et al*., 2012c). In humans, hookworm infections were originally caused by two species, *Ancylostoma duodenale* (Old World Species) and *Necator americanus* (New World Species). The infection is common in tropical and subtropical countries, with *A. duodenale* is reported primarily in Southern Europe, in North and South Asia and parts of Western Australia whereas *N. americanus* is found mainly in Central and Northern South America, Equatorial Africa, Southeast Asia, Polynesia, Micronesia and parts of West Africa.

The diagnostic stage of hookworms is the egg. It is oval in shape, measuring 50 – 60 µm x 35 – 40 µm in size, containing 2 to 8 divided cells (blastomere). It appears as transparent to pale yellowish with thin outer shell under the microscope (Fig 2.3). The favourable temperature for the egg to survive is 28° to 30 °C, although it could still fertile in external temperatures between 10° to 45 °C. The eggs die when the temperature falls below 10 °C. This is the main reason why the infection is restricted to tropical countries. The eggs in the soil rapidly develop into larvae when the environmental conditions are favorable, i.e., availability of oxygen, appropriate humidity and a minimum temperature of 18 °C.

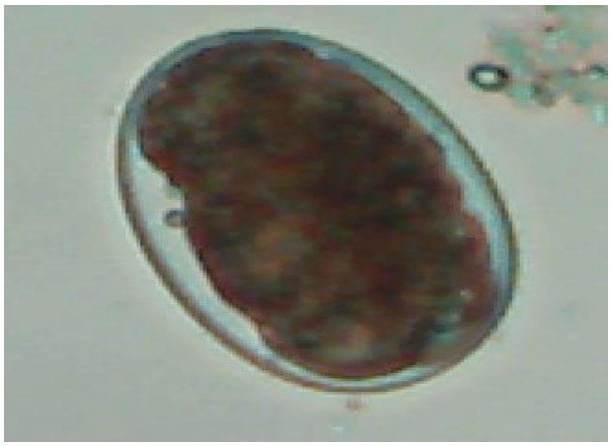

Figure 2.3: Hookworm egg under microscopy (magnification: 100x)

Under favorable conditions of soil, the eggs develop into rhabditiform larvae (first stage, L1) which emerge after 1-2 days. They are free-living larvae with bulbed oesophagus and feed mainly on organic debris and bacteria. The larvae moult on the third day to L2 stage and the bulbed oesophagus disappears on the 5th day. Within 5-6 days, the larvae moult again to form filariform (infective) larvae (L3) with simple muscular oesophagus and a protective sheath. In the soil, the larvae can survive for almost two weeks. However, direct sunlight, salt water and lack of oxygen are fatal to larvae. Hookworm infection is acquired when the L3 larvae penetrate the skin of humans (Fig 2.4). Hookworms can also be acquired through ingestion of contaminated food or geophagia (especially in children). After active cutaneous entry of the filariform larvae into human, the larvae migrate via the circulation to the right side of the heart and from there to the lungs and into the alveoli. They immediately pass through the air passage and ascend the bronchiole, bronchi and migrate via the trachea, epiglottis and from there, the larvae are swallowed into the gastrointestinal tract. The larvae reach the small intestine where they develop into sexually mature adult hookworms. It takes about 3-5 days for the larvae to reach the small intestine after skin penetration. In the intestine, the worm attaches itself to the mucosa of the small intestine via the buccal capsule. In 3-5 weeks, the worms become sexually mature and the female begins to lay fertile eggs.

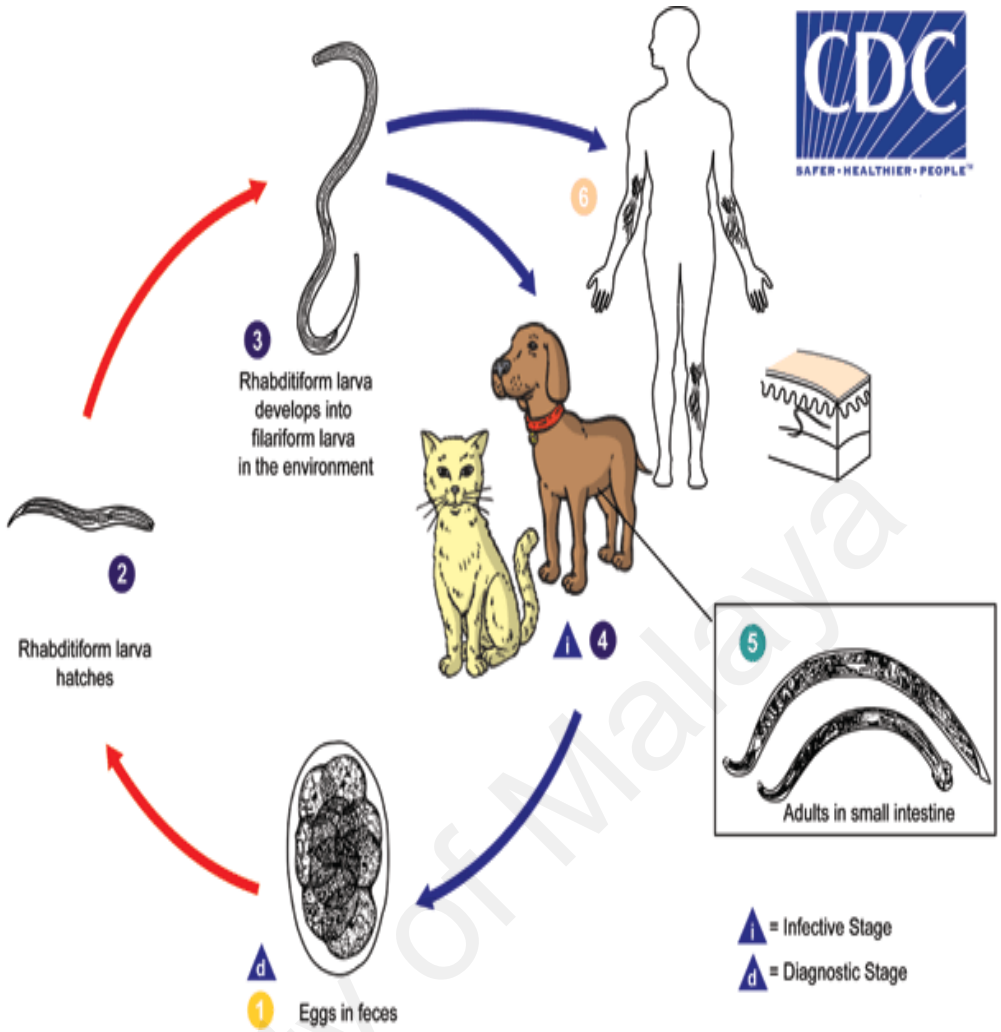

Figure 2.4: Life cycle of *Ancylostoma* species. Source: DPDx (DPDM, 2009)

2.6.1 Clinical manifestations of hookworm infections in humans

The major clinical manifestations of hookworm infection mainly occur due to the chronic intestinal blood loss. Heavy infections can lead to iron-deficiency anaemia and hypoproteinemia. However, the degree of iron-deficiency anaemia induced by hookworms depends on the species, iron content of the diet, the body iron reserve and the extent and duration of the hookworm infection (Albonjco *et al*., 1998). There may also be adverse effects of iron deficiency anaemia (IDA) such as fatigue, nausea, epigastric pain, palpitations, pain in the lower extremities and anorexia affecting the workers‘ productivity (Anyaeze, 2003; Guyatt, 2000). Infant and pre-school children are

particularly vulnerable to the chronic blood and protein loss. Chronic anaemia from hookworms causes deficit in physical growth and fitness, cognitive impairment, reduction in intelligence and memory loss among children (Hotez, 1989; Crompton & Nesheim, 2002; Sakti *et al*., 1999). Additionally, the significant increase in absenteeism, reduced attention and impaired educational performance were reported among school children due to the chronic blood loss (Crompton & Nesheim, 2002). The adverse effects of severe IDA from hookworm infections during pregnancy have been linked to increase maternal mortality, impaired lactation, prematurity and low birth weight (Crompton & Nesheim, 2002; Hotez *et al*., 2010; Hotez *et al*., 2004). In China, vertical transmission of hookworm infection was reported in neonates possibly through ingestion of third-stage larvae in milk and colostrum (Sen-Hai *et al*., 1995).

Infective larvae of hookworms directly penetrate the human skin, especially the hand and feet, resulting in local pruritus, erythematous, papular rash, or "ground itch". In contrast, skin invasion by zoonotic hookworms, *A. braziliense* and *A. caninum*, result in cutaneous larva migrans, or "creeping eruption," a self-limited dermatologic condition characterized by serpiginous burrows (Dhaliwal & Juyal, 2013; Caumes *et al.*, 2002; Miller *et al*., 1991). Hookworms acquired among agricultural labourers is recognized as an occupational hazard due to the handling of human faeces as nightsoil fertilizers (Sawyer, 1923; Brooker & Bundy, 2014; Zhu-xuen & An-xiu, 1993; Bethony *et al*., 2006).

2.6.2 Molecular studies for identification of hookworm species

Microscopic observation for helminth eggs in faecal samples for diagnostic purposes has been used for many decades (Ritchie, 1948). However, it is laborious, time consuming and requires skilled personnel as the eggs are morphologically

indistinguishable from those of other species among hookworm genus. One of the best methods for identification of parasites into species level is through molecular characterization using DNA-based identification. Different species are characterized by distinct DNA sequence which is coded by ribosomal DNA. The rDNA cistron contains both conserved (18S, 5.8S, 28S) and variable regions, ITS-1 and ITS-2. The rDNA cistron also contains external transcribed spacers ETS-1 and ETS-2 and non-transcribed spacers NTS region (Figure 2.5).

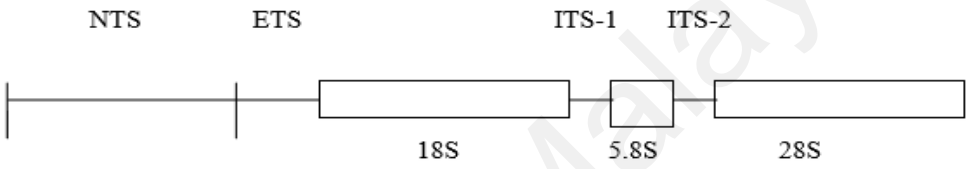

Figure 2.5: Diagram of the ribosomal DNA gene family from Hillis and Davis (1986). The regions coding for the 5.8S, 18S and 28S subunits of rRNA are shown by bars; NTS= non-transcribed spacer, ETS = external transcribed spacer, ITS= internal transcribed spacer regions

In PCR based diagnostic technique, the part of rDNA cistron, especially internal transcribed spacer (ITS) region, is targeted. The internal transcribed spacer (ITS) is sequence of RNA in a primary transcript that lies between precursor ribosomal subunits. Eukaryotic organisms have two internal transcribed spacers which are ITS1 and ITS2. These ITS regions are located between the repeating array of nuclear 18S and 28S ribosomal DNA genes. ITS data has been used in constructing phylogenetic trees, estimating genetic population structures, evaluating population-level evolutionary processes and determining taxonomic identity. Moreover, ITS region is reported to be variable and heterogeneous between parasitic nematode genera and this is useful as an identification tool. The advantage of using this ITS region is due to the high copy number of rDNA, about 30,000 per cell and easy amplification of ITS region (Dubouzet & Shinoda, 1999).

In the last decade, studies have consistently shown that the internal transcribed spacers (ITS-1 and ITS-2) of nuclear ribosomal DNA yield reliable genetic markers for the identification of a broad range of nematodes of the order Strongylida (bursate nematodes) to species (Gasser, 2006; Chilton, 2004; Gasser, 1999; Gasser & Newton, 2000; Chilton & Gasser, 1999; Gasser *et al*., 2004; Matthews *et al*., 2004). With increased demand for identification of hookworms into species level, several techniques, specifically evaluating the genetic markers of ITS1 and ITS2, have been developed (Gasser, 2006). The polymerase chain reaction (PCR) enables selective amplification from complex genomes (Gasser, 2006), and conventional and semi-nested PCR techniques were previously used for characterization of hookworms at molecular levels (Gasser *et al*., 1993).

However, Gasser and Monti (1997) demonstrated the usefulness of PCR-linked single strand conformation polymorphism (SSCP) technique for rapid identification of nematode species. In the experiment, fourteen species of parasitic nematodes (order Strongylida) were characterized by amplification of ITS-2 region of rDNA using PCR. The PCR products were then denatured and subjected to electrophoresis on a non-denaturing gel matrix. SSCP has been used mainly in areas of medical research to analyze genes for sequence variation due to its potential for discrimination of DNA fragments that differ in sequence by a single base (Gasser *et al*., 1998). The product, PCR-SSCP of the single strand of ITS-2, generates distinctive and reproducible patterns for each species allowing rapid delineation of all fourteen species in one step as well as indicating the potential for resolving variation in the ITS-2 sequence within species (Gasser & Monti, 1997).

Later, Gasser *et al*. (1998) and his research team established a mutation scanning approach for ITS region of rDNA using PCR-SSCP technique for the identification of seven hookworm species from different geographical origins. In their experiment, both

ITS-1 and ITS-2 regions of rDNA of seven hookworm species were amplified using PCR; denatured and subjected to electrophoresis in a mutation detection enhancement (MDE) (non-denaturing) gel mix. The electrophoretic mobility of a single-stranded DNA molecule in a non-denaturing gel is dependent on its size and structure (conformations). The conformation(s) are highly dependent on primary nucleotide sequence and a mutation at a particular site in the primary sequence can change the conformation of the molecule, thus altering its electrophoretic mobility. Hence, the mutation scanning method mainly relies on the physical properties of the DNA molecules (Gasser, 1997; Gasser *et al*., 1998). Moreover, Gasser *et al*. (1998) reported the variation in the SSCP patterns for *N. americanus* from different continents (China, Malaysia, Togo and Guatemala) and revealed significant population substructures within the species. This is in accordance with the study by Hu *et al*. (2002), in which he used an SSCP-coupled approach to study haplotypic variability in the mitochondrial cytochrome c oxidase subunit 1 (cox1) within hookworm populations. Genetically distinct groups, *A. duodenale* from China, and *A. caninum* from Australia, were detected which proved the existence of significant population substructures within these species (Gasser *et al*., 2008).

Hu *et al*. (2003) further extended his research by comparing the mitochondrial genome sequence from a *N. americanus* specimens from Togo with one from China, revealing sequence differences of 3-7% and 1-7% (nucleotide and amino acid levels, respectively) in the 12 protein-coding gene (Gasser, 2006; Gasser *et al*., 2008). Other studies have also characterized *Ancylostoma* secreted protein 1 (ASP-1) homologues for *A. caninum*, *A. duodenale* and *N. americanus* (Zhan *et al*., 2003; 2001; Qiang *et al*., 2000;) and Qiang *et al*. (2000) revealed the significant sequence variation (2–3%) at both the DNA and the amino acid levels of *A. caninum* from Shanghai (China) and Baltimore (USA), suggesting that the effect of such molecular variation among

geographically separated parasite populations needed to be considered during the development and evaluation of hookworm vaccines (Gasser *et al*., 2008).

Various fingerprinting methods, such as amplified fragment length polymorphism (AFLP) analysis (de Gruijter *et al*., 2006; Gruijter *et al*., 2005) and restriction fragment length polymorphism (RFLP) analysis (Liu *et al*., 2013; Traub *et al*., 2004) have been applied for exploring genetic diversity and sub-structuring sequence variation among hookworm species population (Gasser, 2006; Gasser *et al*., 2008). Although these approaches are very useful and effective, it can be quite laborious and time consuming to perform post PCR analyses such as electrophoretic analysis and sequencing which are more prone to contamination and the end results on agarose gels could yield no quantitative information.

Verweij *et al*. (2007) evaluated a multiplex real-time PCR assay for the detection and quantitation of DNA of *N. americanus*, *A. duodenale* and *Oesophagostomum bifurcum* in the faeces from infected humans (Gasser, 2006; Gasser *et al*., 2008). However, multiplex real-time PCR using fluorescent detection probes through the possibility of combining assays for the detection of different targets into one reaction has been developed for the diagnosis of hookworm infections in humans (Basuni *et al*., 2011; Verweij *et al*., 2007) and this technique is relatively expensive.

Alternatively, Ngui *et al*. (2012c) described high-resolution melting (HRM) analysis rapid discrimination and identification of human hookworm infection. It is a relatively new post-PCR analysis which does not require complex method or post-PCR procession, allowing direct characterization of PCR amplicons in a closed system and hence has lower risk of contamination (Ngui *et al*., 2012a). Previously, this method has been used in human clinical studies (Radvansky *et al*., 2010; Saitsu *et al*., 2010; Liew *et al*., 2004; Zhou *et al*., 2004; Wittwer *et al*., 2003), molecular studies of parasitic protozoa such as *Leishmania* spp. (Talmi-Frank *et al*., 2010), *Cryptosporidium* spp.

(Pangasa *et al*., 2009), *Plasmodium falciparum* (Andriantsoanirina *et al*., 2009), *Dientamoeba fragilis* (Hussein *et al*., 2009) , *Naegleria* spp. (Robinson *et al*., 2006) and *Giardia* spp. (Bienz *et al*., 2001), rapid discrimination of parasitic worms such as *Brugia malayi* and *B. pahangi* (Areekit *et al*., 2009) and population studies of *Fascioloides magna* (Radvánský *et al*., 2011). With increased demand for identification of the hookworm species among stray cats and dogs in Malaysia, we used real-time PCR accompanied by high resolution melting analysis as it is fast, cost saving, less laborious and has better sensitivity than any molecular methods previously used.

CHAPTER 3: METHODOLOGY

3.1 Study areas

The study was conducted in Klang Valley area in the State of Selangor, Malaysia, comprising Kuala Lumpur, its adjoining cities and suburbs which include Petaling Jaya, Subang Jaya, Kota Damansara, Ampang Hilir, Bangsar, Mont Kiara, Setapak, Wangsa Maju, Jelatak and Kajang. The name Klang Valley was derived from Klang River, one of the longest rivers in Malaysia that flows through Kuala Lumpur, Selangor and eventually flows into the Straits of Malacca. Klang Valley is located in the central-west region of Peninsular Malaysia with the total area of 8347 km^2. The climate is equatorial with hot-humid conditions and rainfall throughout the year.

3.1.1 Selection of the study areas

The study areas fall approximately between latitude 3.139003 and longitude 101.686855 in the central-west region of Peninsular Malaysia. The collection of the faecal samples was carried out in animal shelter houses mainly Paws Animal Welfare Society (PAWS) and Society for the Prevention of Cruelty to Animals (SPCA).

PAWS, a non-governmental organisation founded in 1987, is located at Subang and only 15 km from the city centre of Kuala Lumpur. Currently, it provides shelters and care to approximately 400 homeless animals (mainly cats and dogs). As for SPCA, a well-respected and trusted Non-Profit Organisation, founded in 1958 and currently home to approximately 100 unwanted stray animals, is located at Ampang Jaya, and in the vicinity of 13 km from Kuala Lumpur city. These stray cats and dogs were brought in by the workers (dog-catchers) of Kuala Lumpur City Council.

Meanwhile, the collection of the soil samples were conducted in public places (bus stops, night markets, streets, and children's playgrounds) as well as three recreational parks, including the Kuala Lumpur Convention Centre (KLCC), the Kuala Lumpur Lake Garden and Taman Jaya Park of Petaling Jaya in Selangor (Figure 2.1). The soil samples were collected at places frequented by stray cats and dogs, which can be the source of environmental contamination.

The criteria used in identifying the study areas were;

- Easy access by road
- No history of previous similar study in the area
- Collection of individual faecal sample from the stray cats and dogs that roam the street
- Collection of soil samples from popular tourist attraction places, family recreational places where the risk of parasitic infections from soil is high
- Willingness of the animal shelters' personnels to assist during faecal sample collections.

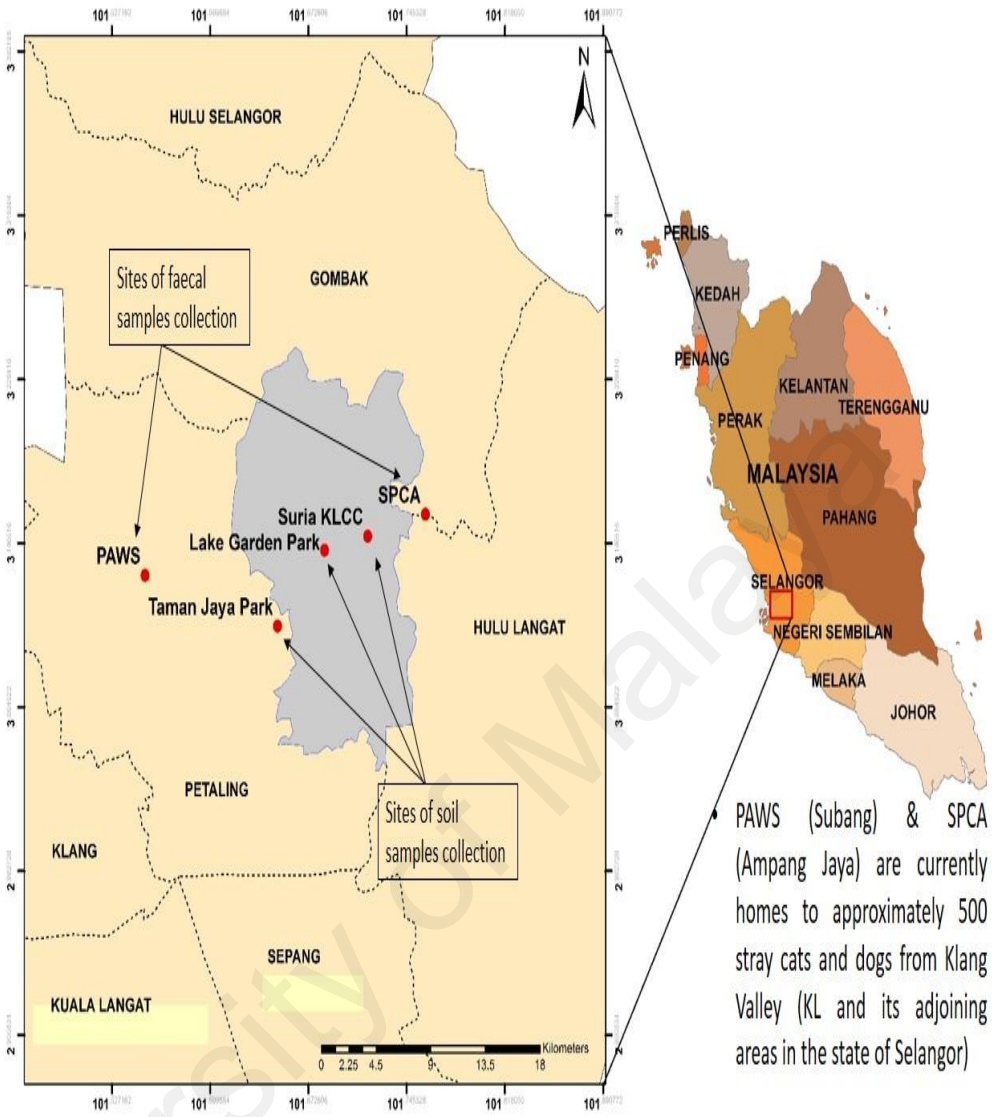

Figure 3.1: Location of the study areas

3.2 Study subjects

The study was conducted among stray and unwanted cats, dogs and soil samples in Klang Valley, Malaysia.

3.2.1 Selection of the study subjects

The main criteria for selection of the study subjects (cats and dogs) was targeting newly arrived cats and dogs every week at PAWS and SPCA so that there is no repetition of collection of the faecal samples from the same cage.

As for the soil samples collection, it was based on the simple random collection from the selected study areas.

3.2.2 Sample size calculation

The sample size required for this study was calculated according to the latest prevalence of intestinal parasitic infection among stray cats and dogs as well as in soil environment. According to the most recent study by Ngui *et al*. (2014), the prevalence of intestinal parasites among cats and dogs was 88.6% The formula used for the calculation of the sample size was according to the following formula (Leedy, 1993) :

$$n \geq (z/m)^2 \times p\,(1-p)$$

Where; n = sample size

z = standard score (1.96)

m = rate of sampling error (5%)

p = estimated rate or case which happen in population

The minimum sample size required for this study was 155 samples, by using the above formula with significance level of 5% and confidence level of 95%. In this study, a total of 505 faecal and soil samples were collected.

3.3 Study Design

A cross-sectional study was conducted among stray cats, dogs and in environmental soil in Klang Valley, Malaysia. The study involves determining the prevalence of intestinal parasites and differentiating hookworms into species levels using molecular analysis.

3.4 Sample collection

Sample collection was carried out over a period of seventeen months, from April 2013 to September 2014. The data collection process is shown in Figure 3.2.

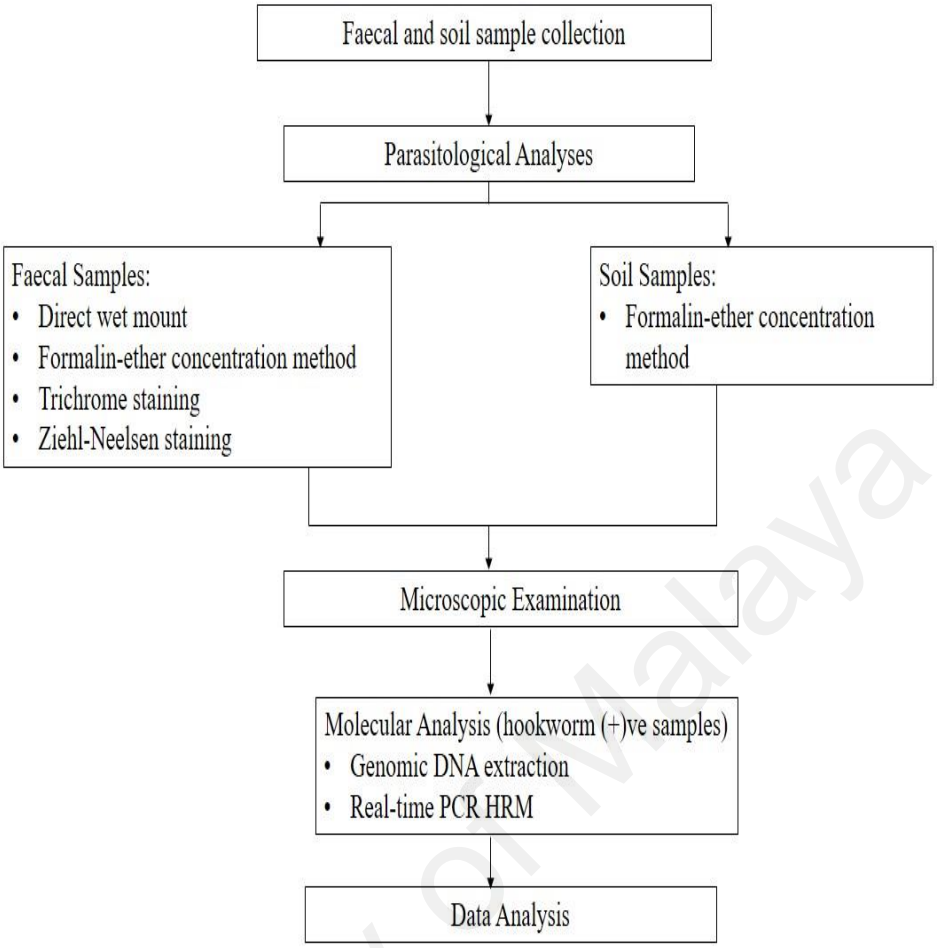

Figure 3.2: Flow chart of the data collection process

3.4.1 Faecal samples collection

The faecal samples were collected in wide mouth screw capped 100 ml clean faecal containers with an attached scoop which were labelled and coded together with a plastic bag. A thumb sized faecal sample was scooped using the provided scoop into the container in order to make sure that the sample was not contaminated with either urine or soil where the animals defaecated. The stray cats and dogs chosen in the current study were those newly targeted by the dog-catchers and that had not been treated by veterinarians. The fresh faecal samples were collected individually in the early morning (06.00 – 08.00) into the labelled containers with the assistance of the animal shelter

workers. The collected samples were transported back to the Department of Parasitology, Faculty of Medicine, University of Malaya, on the same day of collection. Furthermore, half of the faecal sample (from each container) was transferred to a new clean container and preserved with 2.5% potassium dichromate in a ratio of one in three (1:3) parts, respectively. The preserved samples were then stored at 4°C for further analysis.

3.4.2 Soil samples collection

The soil samples were collected from public places (bus stops, night markets, children playgrounds) and three recreational parks, which include Kuala Lumpur Convention Centre (KLCC), the Kuala Lumpur Lake Garden and Taman Jaya in Petaling Jaya, Selangor (Figure 3.1). The soil samples were collected in the morning 06.00 to 08.00 from moist areas within the selected sampling sites. The leaves and debris on the surface of the soil were removed and approximately 200 to 250 g of soil from the surface to 1 cm depth was scraped off using a spoon-screw-capped. The samples were kept in sealed plastic bags, labelled properly with the correct location and transported back to the Department of Parasitology, Faculty of Medicine, University of Malaya, on the same day of collection and stored at room temperature until further analysis. During soil sample collection, the environment of the sample collection sites were observed for cleanliness, sanitation and presence of cats and dogs that can be the source of contamination.

3.5 Parasitological analyses

All samples collected were processed and examined according to the flow chart, Figure 3.2. Direct wet mount preparation, formalin-ether concentration technique, trichrome staining technique and Ziehl-Neelsen staining technique were used for faecal samples in order to detect the intestinal parasites, whereas, only the concentration technique was used for soil samples for identification of intestinal parasites.

3.5.1 Direct wet mount method

The direct wet mount is a routine technique in faecal examination. The technique was used for the preliminary detection of protozoan cysts/oocysts or trophozoites, helminth ova and larvae. The direct wet mount was prepared by mixing a small amount of faecal sample (approximately 2 mg) in a drop of 0.85% saline on a clean, dry microscopic slide. The sample was taken from the stool container using a wooden applicator stick, mixed with saline to form a homogenous suspension and covered with a coverslip. The slide was observed under the microscope and examined on low power (10x magnification). Questionable objects were subjected to further examination using high power (40x and 100 x magnification).

3.5.2 Formalin-ether concentration technique

Formalin-ether concentration technique is one of the important techniques to increase the sensitivity of parasite detection in specimens with light infections and to remove debris. This technique applies the principle of gravity where the specific gravity of protozoan cysts and helminth eggs is greater than that of formalin and ether. Following centrifugation, the heavier parasitic elements will be separated from the

faecal debris and settle to the bottom as a result of gravity. The sediment was placed on a glass slide followed by microscopic examination. For soil samples, the technique was performed with a little modification. The procedure for formalin-ether concentration technique for both faecal and soil samples are listed in Appendices A1 and A2 respectively.

3.5.3 Trichrome staining technique

Trichrome staining is a rapid procedure that gives excellent contrast and visualization of cellular details that aid in the identification of protozoa. In addition, it provides excellent differentiation of internal structures of intestinal parasites as well as facilitating the separation of these organisms from background materials and artefacts. Therefore, this procedure can be used for the confirmation of intestinal parasites detected from direct wet mount preparation and concentration technique. The typical staining reaction with trichrome stain resulted in the cytoplasm of trophozoites and cysts to appear blue-green in colour. Nuclear structure is clearly seen; karyosomal materials of the nuclei and chromatoidal bodies are stained red to purple. The method for trichrome staining is listed in Appendix B.

3.5.4 Ziehl-Neelsen staining technique

The Ziehl–Neelsen staining, also known as the acid-fast staining, is used to identify acid-fast organisms. It is also useful in the identification of some protozoa, such as *Cryptosporidium*. In this technique, *Cryptosporidium* oocysts are stained as bright pink or dark red against a green background. The method of Ziehl-Neelsen staining technique is listed in Appendix C.

3.6 Molecular analysis

The samples (faecal and soil) positive for the hookworm eggs were further processed for molecular analysis in order to differentiate to the species level.

3.6.1 Genomic DNA extraction

DNA extraction was carried out by using PowerSoil®DNA Isolation kit (MO BIO Laboratories, Inc, USA) in which all of the reagents were provided by the manufacturer. The procedures are stated in the manufacturer's protocol and consist of five stages. The first stage was homogenization and cell lysis step, which was started by adding 0.25 grams of samples (faecal and soil) into the provided PowerBead tubes and gently vortexed to mix for a few seconds. The PowerBead Tube contains a buffer that helps to disperse the sample particles, dissolves humic acids and protects nucleic acids from degradation. Then, 60 µl of Solution C1 were added and the tube was inverted several times or vortexed briefly. The Solution C1 contains SDS (an anionic detergent that breaks down fatty acids and lipid associated with the cell membrane of several organisms) and other disruption agents that are required for the complete cell lysis. The PowerBead Tubes were then secured horizontally on a flat-bed vortex pad with tape and vortexed at a maximum speed for 10 minutes. The mechanical shaking introduced by vortexing in the presence of disruption agents and collision of the beads with microbial cells causes the cells to break open and hence the vortexing step is critical for complete homogenization and cell lysis. The PowerBead Tubes were then centrifuged at 10,000 x g for 30 seconds at room temperature. The supernatant was then transferred to a 2 ml Collection Tube provided.

The next stage was the inhibitor removal step, which began when 250 µl of Solution C2 were added to the tube. The mixture was then vortexed and incubated at

4°C for 5 minutes. Solution C2 is patented Inhibitor Removal Technology® (IRT) containing a reagent that can precipitate non-DNA organic and inorganic material including humic substances, cell debris and proteins. The tubes were then centrifuged at room temperature for 1 minute at 10,000 x g and 600 µl of supernatant was transferred to a clean 2 ml Collection Tube provided. Two hundred (200) µl of Solution C3 was then added to the supernatant, vortexed briefly and incubated at 4 °C for 5 minutes. Solution C3 is a patented Inhibitor Removal Technology® (IRT) and is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris and proteins. It is important to remove the contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream DNA applications. The tubes were then centrifuged at room temperature for 1 minute at 10,000 x g and 750 µl of supernatant were transferred to a clean 2 ml Collection Tube provided.

The next step is DNA binding steps in which 1.2 ml of Solution C4 was added to the supernatant and vortexed for 5 seconds. Solution C4 is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this will adjust the DNA solution salt concentrations to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low level. Approximately 675 µl of the mixed solution were then transferred to a Spin Filter and centrifuged at 10,000 x g for 1 minute at room temperature. The flow through was discarded and another 675 µl of supernatant were transferred to the Spin Filter and centrifuged at 10,000 x g for 1 minute at room temperature. The remaining supernatant was loaded onto the Spin Filter and centrifuged at 10,000 x g for 1 minute at room temperature. A total of three loads for each sample processed was required.

The next step was washing steps in which 500 µl of Solution C5 were added to the Spin Filter and centrifuged at room temperature for 30 seconds at 10,000 x g. Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salt, humic acid and other contaminants while allowing the DNA to stay bound to the silica membrane. The flow through, which was just non-DNA organic and inorganic waste removed from the silica Spin Filter membrane by the ethanol wash solution, was discarded. The Spin Filter was then carefully placed back in 2 ml Collection Tube and centrifuged in order to remove residual Solution C5 at room temperature for 1 minute at 10,000 x g. It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests, and gel electrophoresis. The Spin Filter was then carefully placed in a clean 2 ml Collection Tube. The final step was elution step and 50 µl of Solution C6 were added to the center of the white filter membrane making sure the entire membrane was wet. The tube was then centrifuged at room temperature for 30 seconds at 10,000 x g. The Spin Filter was discarded and the DNA in the tube was stored at -20 °C for later use.

3.6.2 HRM-real-time PCR assay

Approximately 180–200 bp within the 5.8S and second internal transcribed spacer (ITS-2) region of the hookworm ribosomal RNA was amplified by real-time PCR using a pair of degenerate primers UMF (Forward: 5‘-CACTGTTTGTCGAACGGYAC-3‘) and UMR (Reverse: 5‘-AGTCSVKRRRCGATTMARCAG-3‘) and then subsequently examined by HRM analysis. The well-defined hookworm genomic DNA for positive controls, *A.*

ceylanicum (GenBank, JQ673421.1) and *A. caninum* (GenBank, JN120882.1) were used. 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/ml of genomic DNA and sterile deionized water using a 7500 Fast real-time PCR system (Applied Biosystems, Inc., CA, USA). The control samples, positive (hookworm DNA) and negative (DNase free water, Sigma Cat. no. W4502) were included in each PCR run. The PCR thermocycling conditions were set according to the optimized protocol at 95°C for 10 min (1 cycle) followed by amplification for 40 cycles consisting of 95°C for 15 sec (denaturation step) and 60°C for 1 min (annealing and elongation steps) (Ngui *et al.*, 2012).

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 sec, 57°C for 1 min (annealing), 95°C for 15 sec (high resolution melting) and final annealing at 60°C for 1 min. In this process, the PCR amplicons were allowed to denature and reanneal before the high resolution melting recording changes in fluorescence with changes in temperature (dF/dT) and plotting against changes in temperature. The high resolution melting curve profile was then analyzed using HRM analysis software version 2.0.1 with fluorescence (melting curve) normalization by selecting the linear region before and after the melting transition. Three different curves were plotted to evaluate the melting characteristics of the two hookworm species. The normalized fluorescence curves formed through the three melting curves (difference plot, derivative melt curve and aligned melt curve) showed distinctively different plots that simply differentiated the two species (Ngui *et al.*, 2012).

3.7 Statistical analysis

Detection of intestinal parasites was determined on the basis of morphological characteristics of specific species (except hookworm where real-time PCR coupled with high resolution melting was performed) under microscopic examination. Each sample was examined and considered positive when the egg or (oo)cyst was observed in each employed technique.

The data entry and statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) software program for Windows version 2.2 (SPSS, Chicago, IL, USA). Descriptive statistics were mainly used to describe the prevalence of intestinal parasites in which proportion (percentage) was used. Pearson's chi-square (χ^2) was carried out to test the differences of intestinal parasitic infections based on egg or cyst/oocyst passed out among stray cats and dogs. The level of statistical significance was set at $p < 0.05$ and for statistically significant factor, an odds ratio (OR) and 95% confidence interval (CI) were computed.

3.8 Ethical consideration

The protocol of the current study was reviewed and approved by the Institutional Animal Care and Use committee of the University of Malaya, Kuala Lumpur [Ethics Reference number: PAR/29/06/2012/II (R)]. Written permission was additionally obtained from the management authorities of the SPCA. Authorities of PAWS agreed verbally without any written permission. No permission was required for collection of soil samples as the data collection was not primarily for research but for the public health information. It was confirmed that our study did not involve endangered or protected species. The objectives and protocols of the research were thoroughly discussed with the authorities in charge.

CHAPTER 4: RESULTS

4.1 The overall prevalence of intestinal helminths and protozoa detected by microscopy

The total number of faecal samples examined were 379, with 227 (60%) from stray dogs and 152 (40%) from stray cats. Of the 379 faecal samples examined, 287 (75.5%; 95% CI = 71.2%-79.9%) were positive with at least one helminth species and 77 (20.3%; 95% CI = 16.4%-24.3%) were with protozoa based on excreted eggs and/or cysts/oocysts, respectively. The prevalence of helminth eggs among stray dogs (87.7%) was significantly higher ($p<0.0001$, $x^2= 43.9$) than those of cats (57.9%). As for protozoa, the prevalence among stray dogs (18.9%) was significantly lower ($p<0.001$, $x^2=144.3$) than that of stray cats (22.4%). Hence stray dogs have higher chance of carrying intestinal helminths and stray cats have higher potential of carrying intestinal protozoa (Table 4.1).

Table 4.1: Total prevalence of intestinal parasites (helminths and protozoa) among stray cats and dogs

Animals	Helminths				Protozoa		
	N	n	%	95%CI	n	%	95%CI
Cats	152	88	57.9	50 - 65.8	34	22.4	15.1-28.9
Dogs	227	199	87.7	83.3 - 91.6	43	18.9	14.1-24.2

Total	379	287	75.7	71.2 - 79.9	77	20.3	16.4-24.3

N = Total number of faecal samples collected; n = infective number of faecal samples

4.2 Prevalence of gastrointestinal parasites by species in stray cats and dogs

Both dogs and cats were infected with five genera of helminths, including hookworms (46.4%, 176/379), *Toxocara* spp. (11.1%, 42/379), *Trichuris* spp. (8.4%, 32/379), *Spirometra* spp. (7.4%, 28/379) and *Ascaris* spp. (2.4%, 9/379). As for protozoa, four genera were reported including *Giardia* spp. (8.2%, 31/379), *Cryptosporidium* spp. (4.5%, 17/379), *Isospora* spp. (3.4%, 13/379) and *Cyclospora* spp. (4.2%, 16/379).

In stray cats, infection of hookworm spp. (36.2%) was the highest followed by *Spirometra* spp. (14%) and *Toxocara* spp. (9.9%). The remaining helminths identified were *Trichuris* spp. (2.0%) and *Ascaris* spp. (0.7%). The most frequently observed protozoa were *Giardia* spp. (7.2%) and *Cryptosporidium* spp. (6.6%), followed by *Cyclospora* spp. (4.6%), and *Isospora* spp. (3.9%). As for stray dogs, the highest prevalence was the hookworm infections (53.3%), followed by *Toxocara* spp. (11.9%), *Trichuris* spp. (11%), *Ascaris* spp. (7.9%) and *Spirometra* spp. (3.5%). The most common protozoan in dogs was *Giardia* spp. (8.8%). The remaining protozoa infections were *Cyclospora* spp. (4%), *Cryptosporidium* spp. and *Isospora* spp. (3.1% each). The presence of helminth eggs and protozoa cysts/(oo)cysts in faecal samples among stray cats and dogs are summarized in Table 4.2.

Table 4.2: Prevalence of intestinal parasites by species in faecal samples of stray cats and dogs

Parasites	**Stray cats**		**Stray dogs**	
Helminths	n=152	% infected	n=227	% infected

1. hookworms	55	36.2	121	53.3
2. ***Toxocara***	15	9.9	27	11.9
3. ***Trichuris***	3	2.0	25	11.0
4. ***Ascaris***	1	0.7	18	7.9
5. ***Spirometra***	14	14	8	3.5
Protozoa				
1. ***Giardia***	11	7.2	20	8.8
2.***Cryptosporidium***	10	6.6	7	3.1
3. ***Isospora***	6	3.9	7	3.1
4. ***Cyclospora***	7	4.6	9	4.0

n = total number of animals' faecal samples collected

4.3 Prevalence of intestinal parasites detected in soil environments

Among the soil samples, 23% [29/126 (95% CI = 15.1%–31%)] contained helminth eggs including hookworms (16.6%, 21/126), *Ascaris* spp. (4%, 5/126) and *Toxocara* spp. (2.4%, 3/126). Moreover, 9.5% [12/126 (95% CI = 4.8%-14.3%)] of soil samples were found to be contaminated with two genera of protozoa with *Isospora* spp. (7.1%) being the commonest protozoa followed by *Giardia* spp. (2.4%).

4.4 Identification of hookworm species using real-time PCR–HRM analysis

All 197 microscopically positive samples for hookworm eggs (55 cats, 121 dogs, and 21 soil samples) were successfully amplified by real-time PCR accompanied with high resolution melting analysis. Only two *Ancylostoma* spp., *A. ceylanicum* and *A. caninum*, were detected, as seen in Table 4.3. *A. ceylanicum* was detected to be dominant in samples from stray cats (29.6%, 45/152), dogs (44.5%, 101/227) and soil (14.3%, 18/126) as compared with *A. caninum*. Only single species, either *A. ceylanicum* or *A.caninum* (no mixed infection), was detected in the current study.

Table 4.3: Prevalence of *Ancylostoma* species in faecal and soil samples as detected

by real-time polymerase chain reaction–high resolution melting analysis

Hookworm species	No (%) of positive by PCR-HRM
1. Cat samples (n = 152)	
A. ceylanicum	45 (29.6)
A. caninum	10 (6.6)
2. Dog samples (n = 227)	
A. ceylanicum	101 (44.5)
A. caninum	20 (8.8)
3. Soil samples (n = 126)	
A. ceylanicum	18 (14.3)
A. caninum	3 (2.4)

A = *Ancylostoma*; n = total number of sample collected

4.5 *Ancylostoma* species based on HRM curve profile

The melting characteristics of ITS-2 amplicons from *A. ceylanicum* and *A. caninum* were assessed by plotting three different curves (Figures 4.1, 4.2, 4.3). In the present study, the normalized fluorescence curves, i.e., aligned melt curve (Figure 4.1), derivative melt curve (Figure 4.2) and difference plot melt curve (Figure 4.3) produced uniquely different plots that were easily distinguishable for the two species.

Although the melting temperature patterns for the two species were similar, they could be clearly differentiated by melting curves as shown in Figures 4.1 - 4.3. For each of the hookworm species, a sharp decreased in fluorescence was detected in denatured DNA as shown in normalized fluorescence curves (Figure 4.1), which was consistent with its respective melting profile (Figure 4.2).

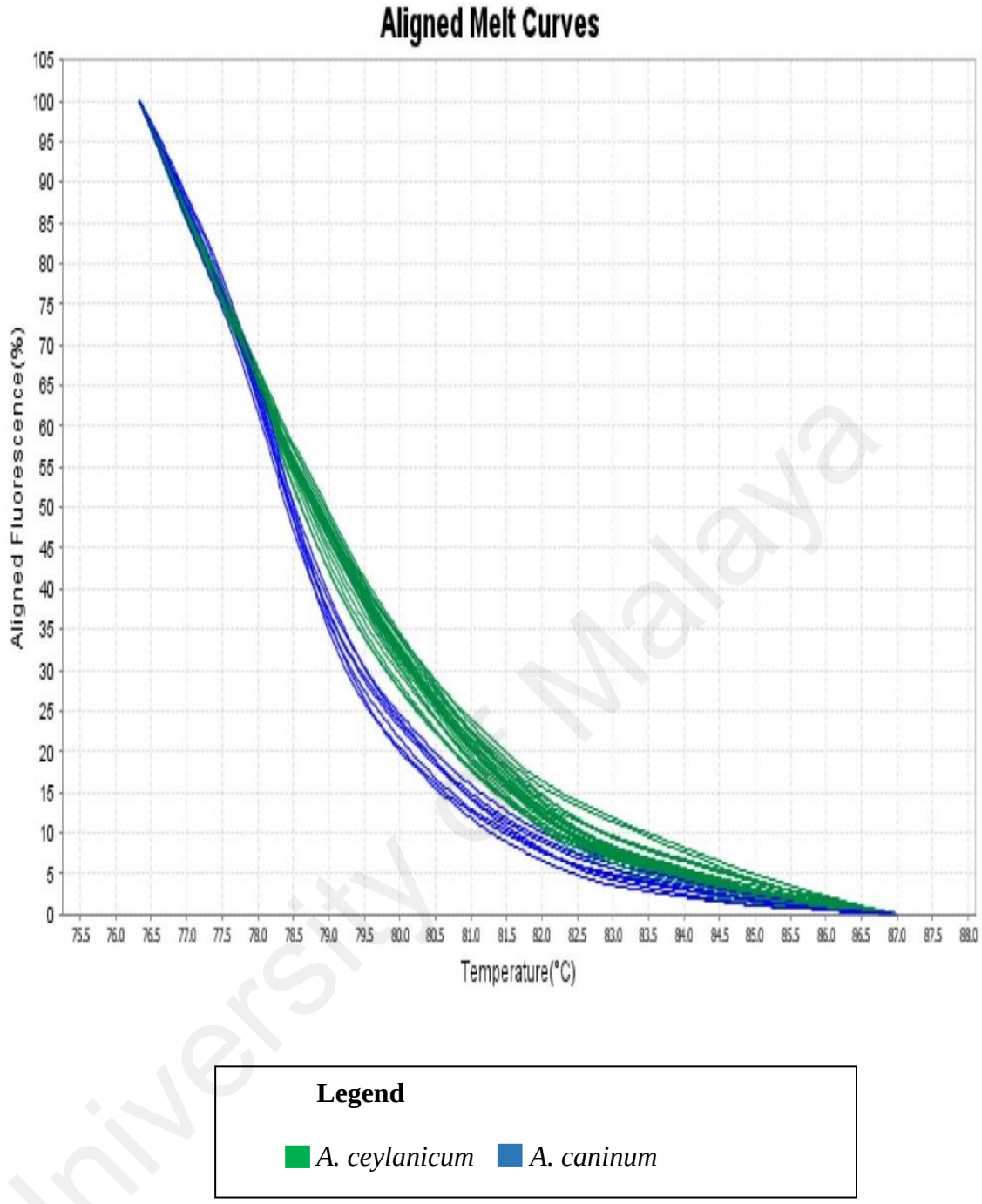

Figure 4.1: Representative profiles of the melting curves (aligned melt curves) of ITS-2 amplicons for *Ancylostoma ceylanicum* (green), and *A. caninum* (blue). Fluorescence is plotted against degrees Celsius (°C). The curve included hookworm spp. from different hosts i.e., cats, dogs and soil samples

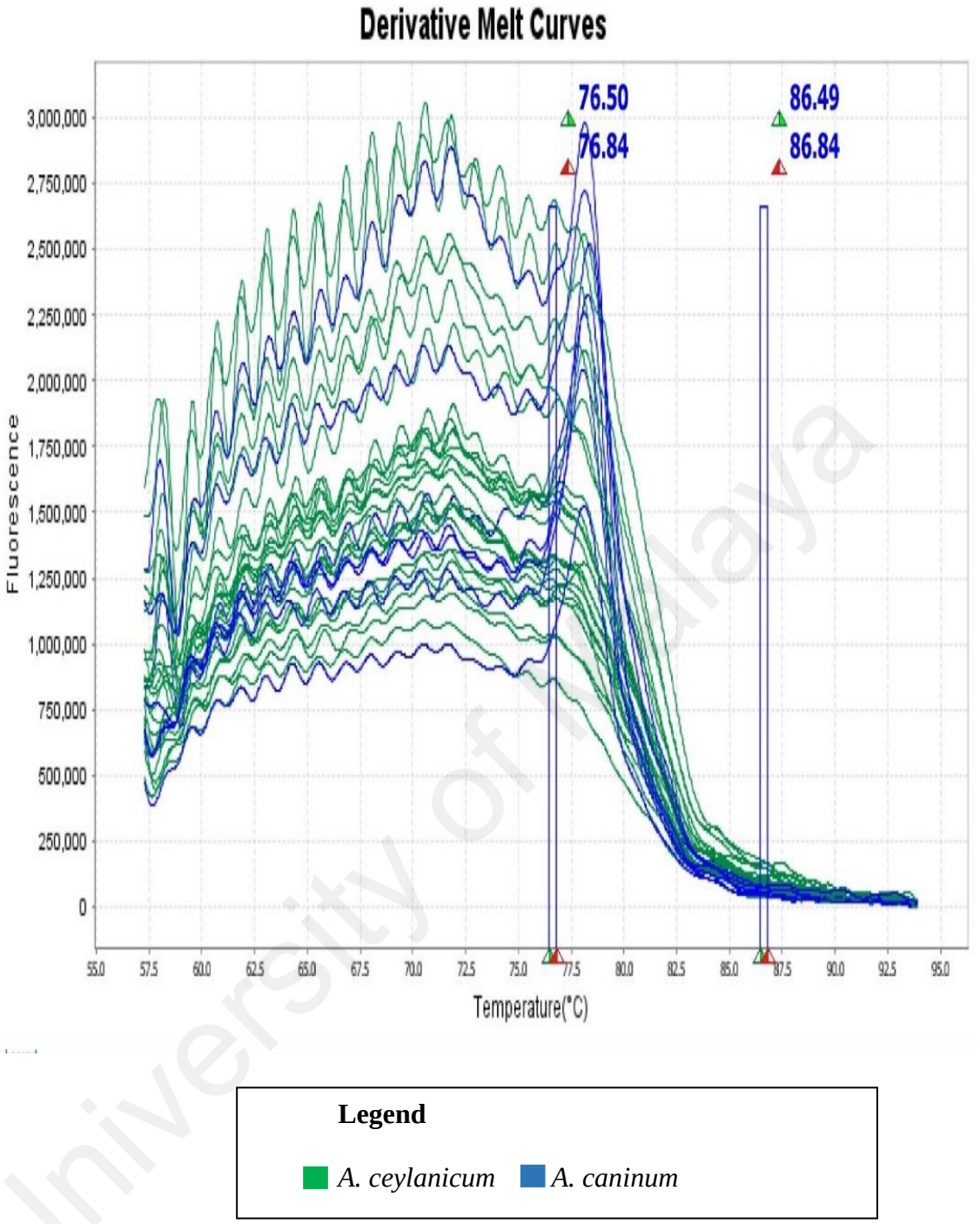

Figure 4.2: Representative profiles of the melting curves (derivative melt curves) of ITS-2 amplicons for *Ancylostoma ceylanicum* (green), and *A. caninum* (blue). It is noted that *Ancylostoma* spp. produced single peak. The curve included hookworm spp. from different hosts i.e., cats, dogs and soil samples

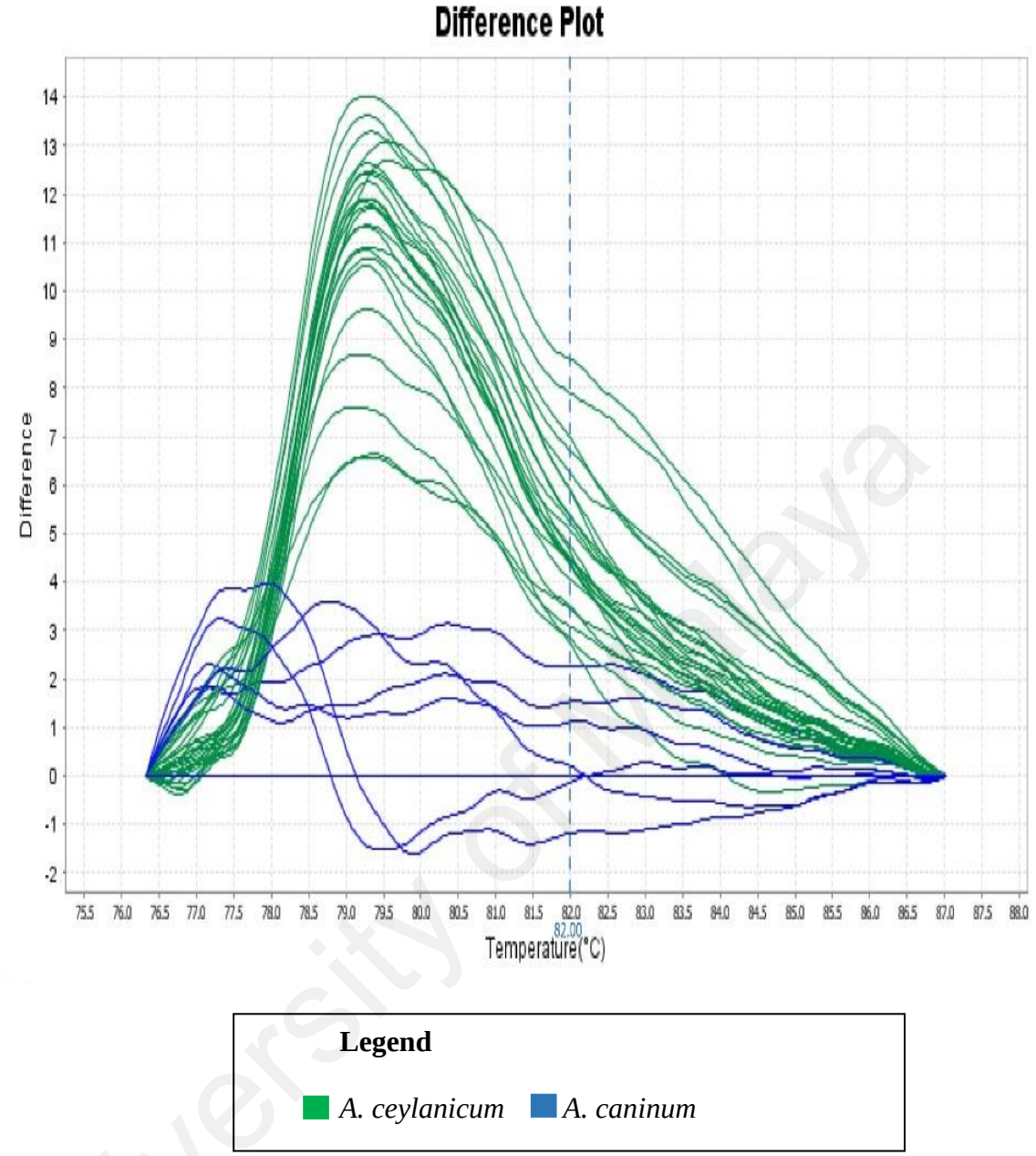

Figure 4.3: Representative profiles of the melting curves (difference plot curves) of ITS-2 amplicons for *Ancylostoma ceylanicum* (green), and *A. caninum* (blue). The curve included hookworm spp. from different hosts i.e., cats, dogs and soil samples

CHAPTER 5: DISCUSSION

The sampling sites selected in the current study are situated in Klang Valley, the most developed region in Malaysia. According to the 2010 population and housing census, Klang Valley is home to approximately 7 million people (DOS, 2006) including a large number of local migrants from other states in Malaysia as well as foreign workers particularly from Indonesia, Nepal, Myanmar, India, and Bangladesh. Additionally, this region is also home for many stray cats and dogs that roam public places, such as parks, night markets, street food-stalls, open restaurants and housing areas. Most of these animals are not sterilised, are left to reproduce, and therefore, the number of unwanted animals far exceeds the number of adoptions. Occasionally, these animals are captured by city council workers (dog-catchers) and are sent to animal welfare shelters (e.g. PAWS and SPCA). However, many remain in public areas and could potentially harbour zoonotic parasites. The results of the current study provide an insight into the zoonotic helminths and protozoa that occur in domestic environments, potentially due to faecal contamination by infected stray cats and dogs.

Based on microscopic observation on a single faecal collection, 75.5% of the samples were found to contain helminth eggs (57.9% in cats and 87.7% in dogs). The eggs were mostly of nematodes [hookworms (46.4%), *Toxocara* spp. (11.1%), *Trichuris* spp. (8.4%) and *Ascaris* spp. (2.4%)] and one cestode [*Spirometra* spp. (7.4%)] worm. This finding is in agreement with other studies of cats and dogs, which noted a prevalence of 61.9% and 88.6% (Ngui *et al*., 2014; 2012b) respectively, in Malaysia, and these infections were dominated by nematode worms (Ngui *et al*., 2014; Zain *et al*., 2013). Moreover, it was observed that the prevalence of helminthes among stray cats can be as high as 94% as described by Hajipour *et al*. (2015) in Iran or 90.7% by Waap *et al*. (2014) in Portugal which were in contrast with the finding in Thailand (11.9%)

(Jittapalapong *et al*., 2007a). This major variation in prevalence may be due to the geographical distribution of helminths, and the variation in the natural environmental conditions that provides the survival of helminth parasites.

On the other hand, in other countries, stray cats were reported to be mainly infected with cestode worms, comprising 97.6% of the total parasite load in Qatar (Abu-Madi *et al*., 2010) and 53.1% in Portugal (Waap *et al*., 2014). In addition, more than 80% of reported cases of human cestodes are from East and South East Asian countries including China, India, South Korea, Thailand and Japan which are mainly due to the unusual habits of eating raw fish and ingesting the second intermediate hosts such as snakes, frogs and birds (Liu *et al*., 2015; Sabu *et al*., 2014; Duggal *et al*., 2011; Nithiuthai *et al*., 2004). Therefore, it is suggested that Malaysian stray cats and dogs are more prone to infections with intestinal nematodes from contaminated environmental soil.

From the result of the present study, it is evident that helminth infections (75.5%) are more common than protozoa infections (20.3%) in both cats and dogs. This finding is in agreement with previous studies on faecal samples of cats and dogs (Yamamoto *et al*., 2009; Lorenzini *et al*., 2007; Meloni *et al*., 1993; Vanparijs *et al*., 1991). In contrast, other studies have emphasized on increasing prevalence of zoonotic protozoa such as *Giardia* spp. and *Cryptosporidium* spp. (Smith & Grimason, 2003; Uehlinger *et al*., 2013; Savioli *et al*., 2006). The present study also revealed that stray cats have higher potential of carrying intestinal parasitic protozoa whereas stray dogs have higher chance of carrying intestinal helminths.

Further statistical analysis revealed that there was significantly ($x^2 = 43.89$, $p < 0.001$) more stray dogs (87.7%) being infected with helminths than stray cats (57.9%), among which hookworms was the most prevalent helminth. Similar

observations were additionally reported in several surveys among cats and dogs, including in Thailand (Traub *et al*., 2008; Inpankaew *et al*., 2007), Cambodia (Inpankaew *et al*., 2014), India (Traub *et al*., 2014), Brazil (Lorenzini *et al*., 2007), Venezuela (am re -Barrios *et al*., 2004) and Costa Rica (Scorza *et al*., 2011).

Subsequently, *Toxocara* spp. (11.1%) was the second most prevalent in faecal samples from both stray cats and dogs. Among dogs, a prevalence of 11.9% was found (*T. canis*) and 9.9% prevalence was identified among cats (*T. cati*). These results showed that the prevalence of *Toxocara* spp. in the current study was higher than that reported among cats and dogs in Thailand (3.5%) (Jittapalapong *et al*., 2007a), India (4%) (Krecek *et al*., 2010) and Brazil (5.5%) (Oliveira-Sequeira *et al*., 2002). In particular, among faecal samples of cats, our results were similar to that of a report from Egypt (9%) (Khalafalla, 2011) but contrasted with reports from Denmark (79%) (Engbaek *et al*., 1984), Iran (78%) (Hajipour *et al*., 2015) and Spain (55.2%) (Calvete *et al*., 1998).

The third most common helminth detected in the current study was *Trichuris* spp., found to be higher among stray dogs (11 %) as compared to stray cats (2%) which was in agreement with a previous study in Malaysia (Ngui *et al*., 2014). *Trichuris* spp. in cats in the current study was noted to be higher than in Japan (0.2%) (Yamamoto *et al*., 2009) but lower than those in West India (71%) (Krecek *et al*., 2010). Due to the similarities in morphology, *Trichuris* eggs detected in the current study represent either *T. trichiura* or *T. vulpis*, which requires further molecular identification into species level to identify the species.

Ascaris spp. eggs that resemble *A. lumbricoides* (human origin) were detected in samples from stray dogs (7.9%), cats (0.7%) and soil (4%). Stray dogs were reported to be the environmental contaminators of *Ascaris* spp. eggs around the communities

(Shalaby *et al*., 2010). Furthermore, the DNA extracted from *Ascaris* spp. eggs from dogs was found to be of 100% homology to those of *A. lumbricoides* obtained from humans (Traub *et al*., 2003). Therefore, the current finding may be due to environmental contamination in which stray cats and/or dogs, probably acting as carriers of ascariasis in human populations.

The eggs of *Spirometra* spp. were found in faecal samples of both stray cats (14%) and dogs (3.5%). In the previous study performed in Malaysia, *Spirometra* spp. was noted to be absent among stray cats (Zain *et al*., 2013), and surprisingly, the prevalence of *Spirometra* spp. found in the current study was higher than that in cats reported from Shiraz, Iran (3.8%) (Zibaei *et al*., 2007) and New York, USA (0.4%) (Lucio-Forster & Bowman, 2011). This could be due to the characteristic nature of stray cats, which tend to eat infected paratenic hosts such as fish, frogs and birds from the surroundings. When the carnivorous mammalian hosts (cats and dogs) eat infected second intermediate hosts, the larva mature and develop into adult, producing eggs (Mueller, 1966). Humans are accidental intermediate hosts acquiring infections mainly due to (a) the consumption of raw or undercooked infected meat of frogs or snakes or (b) through drinking untreated water containing infected copepods with infective larva or (c) the unusual usage of treating open wounds or lesions with poultices using infected frogs or snakes flesh (Sabu *et al*., 2014; Li *et al*., 2011).

The detection of helminths in the infected hosts, solely based on the finding of eggs in faecal samples may have disadvantages such as being missed or undetectable in cases of low infections. The detection of infection by post-mortem has the advantage over faecal-egg examination due to the accessibility of the helminths directly from the animals. However, it is unethical to sacrifice animals to detect the helminths. Thus, the degree of helminth infections in the current study may be higher than currently reported,

as only infections by matured helminths that are capable of producing eggs were counted.

Four protozoa genera encountered in this study were *Giardia, Cryptosporidium, Isospora* and *Cyclospora.* As for *Toxoplasma gondii*, its oocyst was not detected in the cats' faeces in this study. According to Dubey *et al*. (1970), cats only shed *T. gondii* oocysts once during their lifetime. This could be the reason for not detecting *Toxoplasma* oocysts. *Giardia* spp. was the most frequently found protozoa among cats (7.2%) and dogs (8.8%) in this study. Similar result was reported from Thailand whereby 7.9% prevalence of *Giardia* spp. were detected among dogs in temple communities and this could pose as a potential zoonotic risk to humans for transmission of *Giardia* (especially Assemblage A genotype) (Inpankaew *et al*., 2007). Barutzki and Schaper (2011) reported that among 24,677 dogs and 8560 cats examined, 18.6% of dogs and 12.6% of cats were reported to be infected with *Giardia* spp. in Germany between 2003 and 2010, and the higher prevalence rates were found in younger age of 3 to 6 months old (Barutzki & Schaper, 2011). In contrast, the prevalence of *Giardia* spp. detected among cats and dogs were as low as 0.7% in Romania (Mircean *et al*., 2010), 0.9% in Japan (Yamamoto *et al*., 2009) and 2% in Egypt (Khalafalla, 2011). Increase prevalence of *Giardia* in animals is correlated to the possibilities of soil being contaminated with the faeces of infected stray cats and dogs (Barwick *et al*., 2003).

Cryptosporidium spp., was the second most common gastrointestinal (GI) protozoa reported in the present study (6.6% in stray cats and 3.1% in stray dogs). The current findings were higher than those reported among cats and dogs in California (2%) (El-Ahraf *et al*., 1991), Brazil (2.41%) (Huber *et al*., 2005), Greece (2.8%) (Papazahariadou *et al*., 2007), Bangkok (1.5%) (Inpankaew *et al*., 2007) and Japan (0.2%) (Yamamoto *et al*., 2009). Meanwhile, Lucio-Forster *et al*. (2010) reported that

the prevalence rate ranges of *Cryptosporidium* spp. to be 0-29.4% among cats and 0.5-44.4% among dogs. The role of cats and dogs as zoonotic potential and important reservoirs for *Cryptosporidium* spp. is well recognised (Uehlinger *et al*., 2013; Thompson *et al*., 2008; Xiao *et al*., 2004; Robertson *et al*., 2000). To date, there are 16 different species of *Cryptosporidium* and over 40 genotypes that have been recognized, with new genotypes regularly being identified (Ngui *et al*., 2014; Ng *et al*., 2008).

For *Isospora* spp., the prevalence rates are 3.9% and 3.1% for both stray cats and dogs, respectively in the current study. The prevalence of *Isospora* spp. among stray cats is comparable to those in Egypt (2%) (Khalafalla, 2011) but lesser than Portugal (14.2%) (Waap *et al*., 2014), Venezuela (8.1%) (am re -Barrios *et al*., 2004), Romania (11.6%) (Mircean *et al*., 2010), Spain (22%) (Martínez-Moreno *et al*., 2007), Thailand (10%) (Inpankaew *et al*., 2007), Greece (3.9%) (Papazahariadou *et al*., 2007) and Germany (5.6%) (Barutzki & Schaper, 2011). Its prevalence was high in younger cats, less than 1 year old (Kirkpatrick, 1988; Visco *et al*., 1977; Chiejina & Ekwe, 1986; Mircean *et al*., 2010; am re -Barrios *et al*., 2004; Hoskins *et al*., 1982; Lightner *et al*., 1978). Dogs were reported to be the hosts for *I. canis*, *I. ohioensis*, *I. neorivolta* and *I. burrowsi* while cats were hosts for *I. felis* and *I.* rivolta (Lappin, 2010; Dubey, 2009; Matsubayashi *et al*., 2011). Infections in dogs and cats were due to the ingestion of sporulated oocysts carried by paratenic hosts such as flies, cockroaches or dung beetles (Buehl *et al*., 2006).

In soil, *Isospora* spp., was found to be the commonest protozoa (7.1%) which was comparable with the study done in public places of Tehran, Iran (10.7%) (Tavalla et al., 2014). Moreover, Tavalla *et al*. (2014) reported that *Isospora* spp. had animal origins and were capable of contaminating the environment through faeces of dogs, cats

and birds. In addition, *Isospora* spp. oocysts were highly resistant to the environmental harsh conditions (Lappin, 2010).

In humans, *I. belli* was responsible for human intestinal isosporiasis (Lindsay *et al*., 1997). Most of the reported cases in humans occurred in the tropics rather than in the temperate zone and were widely spread across Asia, South America and Africa (Soave & Johnson, 1988; Jongwutiwes *et al*., 2007; Lindsay *et al*., 1997). Infections were also reported among children and travellers to tropical regions (Goodgame, 2003; Rodriguez-Morales & Castañeda-Hernández, 2014). *Isospora* spp. is also regarded as an opportunistic enteric pathogen among AIDS patients (Williams *et al*., 2011; De-Horvitz *et al*., 1987). Clinical features of isosporiasis in humans include prolonged diarrhoea, steatorrhoea, headache, fever, weight loss, abdominal pain, dehydration, malaise and fever. Persistent diarrhoea was also reported among travellers from Europe (Goodgame, 2003). If the diarrhoea is severe, potassium, sodium and chloride concentrations may be decreased due to gastrointestinal losses (Lappin, 2010). Moreover, infections of intestinal epithelium can result in intestinal inflammation (enterocolitis). Symptoms are severe among immunodeficient patients, infants and old people (Williams *et al*., 2011; ten Hove *et al*., 2008). Therefore, the spectrum of isosporiasis seems to be variable, partly depending on the host immune status whereas the virulence of parasite remains unknown (Jongwutiwes *et al*., 2007).

Another coccidian found in this study was *Cyclospora* spp. with 4.6% among stray cats and 4% stray dogs. Although *Cyclospora* spp. was rarely reported among domestic animals, outbreak of cyclosporiasis has been reported in many countries including US (Berlin *et al*., 1994), UK (Clarke & McIntyre, 1996), Cuba (Escobedo & Núñez, 1999), Egypt (Rizk & Soliman, 2001), Venezuela (Chacin-Bonilla *et al*., 2001), Zimbabwe (Gumbo *et al*., 1999), Nigeria (Alakpa & Fagbenro-Beyioku, 2002) and

Bangladesh (Albert *et al*., 1994). In addition, it has been reported in fresh fruits, green-leafy vegetables, and edible shellfish in the markets (Goodgame, 2003; Aksoy *et al*., 2014; Ortega *et al*., 1997). The oocysts are resistant to the harsh conditions in the environment and only a few number of oocysts (as low as 10 oocysts) are needed for the human infections (Adam & Ortega, 1999). *C. cayetanensis* is the only species known to infect humans, particularly the mucosal epithelium of the intestine or bile ducts (Percival *et al*., 2004; Ortega *et al*., 1997). The mode of transmission for human infections is faecal-oral route through contaminated food and water from the contaminated environment or related to international travel to the endemic areas (Chalmers, 2014; Goodgame, 2003). It often causes diarrhoea in endemic areas and has been mainly described as the cause of travellers' diarrhoea (Goodgame, 2003; Puente *et al*., 2006). Other symptoms include anorexia, nausea, cough, purulent sputum, vomiting, abdominal bloating and cramps, weight loss, fatigue, low grade fever and body aches (Percival *et al*., 2004). Asymptomatic infections are also common in endemic regions (Chalmers, 2014). Although the disease is self-limiting, the symptoms are prolonged and severe among immnocompromised (HIV/AIDS) patients (Pape *et al*., 1994). Some complications after cyclosporiasis are Guillain-Barré syndrome (Richardson *et al*., 1998), reactive arthritis (e iter's syndrome) (Connor *et al*., 2001) and biliary tract infections leading to acalculous cholangitis and cholecystitis (de Górgolas *et al*., 2001; Zar *et al*., 2001; Sifuentes-Osornio *et al*., 1995).

Related to the environmental soil, 23% of the samples were found to be positive for helminth eggs, which was in accordance with the previous studies in Malaysia (26.7%) (Azian *et al*., 2008) and Montreal (25.6%) (Ghadirian *et al*., 1976), but lower than in Turkey (84.4%) (Ulukanligil *et al*., 2001). Variations in the distribution of these helminths is highly dependent on the climate and the environment factors that favour the survival of the helminth eggs, the environmental hygiene and exposure to the

susceptible animals. Since Malaysia is a tropical country with hot and humid weather, the transmission of helminths from the soil is favourable, particularly in the areas where stray cats and dogs are common. In this study, we found 16.6% hookworms, 4% *Ascaris* spp. and 2.4% *Toxocara* spp. Contamination with *Toxocara* eggs in soil samples from public areas were noted in various countries, such as Spain (36.4%) (Martínez-Moreno *et al*., 2007), Italy (63.6%) (Giacometti *et al*., 2000), Turkey (30.6%) (Oge & Oge, 2000) as well in Malaysia (12.1%) (Azian *et al*., 2008). *Toxocara* spp. was reported to be the one of the major parasites contaminating the soil, as the adult females worms are capable of producing up to 200,000 eggs per day (Schnieder *et al*., 2011). The contamination of soil by infective *Toxocara* eggs was noted to be proportional with the prevalence of human toxocariasis; hence, *Toxocara* spp. was noted to be among the important parasites in public health (Mizgajska, 2001). However, *Toxocara* eggs found in the current study can additionally be from another new variant of *Toxocara* species, i.e *T. malaysiensis* (Li *et al*., 2006; Gibbons *et al*., 2001). Consequently, cross-infection might have occurred among these stray animals, although *T. canis* and *T. cati* are known to be dog and cat nematodes, respectively.

The identification of helminths based on the eggs morphology can be satisfied up to the genus level, and the method has been used within parasitology laboratories for diagnosis purposes for many decades. Morphological observations coupled with molecular techniques have been found to be the best methods to identify the parasite species to date. Thus, our subsequent report included the identification of hookworm species (the highest prevalence in both faecal and soil samples) using real-time PCR coupled with high resolution melting analysis. All samples from dogs (121), cats (55) and soil (21) that were microscopically positive for hookworm eggs were successfully analyzed revealing two species, namely *A. ceylanicum* and *A. caninum*, in which *A. ceylanicum* was found to be more prevalent and respectively dominated in dogs

(101/121, 20/121), cats (45/55, 10/55) and soil (18/21, 3/21) samples. In addition, several previous studies reported that *A. ceylanicum* was dominant in dogs (Ngui *et al*., 2012b; Choo *et al*., 2000) and humans from Malaysia (Ngui *et al*., 2012c) and suggested that humans are at risk of zoonotic *A. ceylanicum* infections from dogs (Mahdy *et al*., 2012).

On the other hand, *A. caninum* that is known as one of the canine hookworm, was detected not only in stray dogs but also in stray cats, with this being the first such report in Malaysia to date. This finding has demonstrated that there was a cross-infection of *A. caninum* between cats and dogs in the studied areas and cats are now the victims of dog hookworms, which may be due to the coexistence nature of cats and dogs in the communities. Our result was in agreement with previous findings in China (Liu *et al*., 2013), Australia (Palmer *et al*., 2007) and Thailand (Setasuban *et al*., 1976), where *A. caninum* species were found among cats. Despite *A. braziliense* being noted among cats in Malaysia (Zain *et al*., 2013; Ngui *et al*., 2012b), none were detected in the current study.

5.1 Control and prevention of zoonotic intestinal parasites

The role of companion animals as reservoirs for zoonotic diseases has been known as a significant health problem worldwide (Schantz, 1994; Ngui *et al*., 2012a). Therefore, control and prevention of the zoonotic diseases are necessary tasks that require the integrative and multidisciplinary approaches (Chomel, 2008). Rational measures for preventing zoonotic infections in humans include pet owner education, and regular deworming of cats and dogs (Guillot & Bouree, 2007). Public health education, the risk factors of parasitic infections as well as veterinary awareness are also necessary due to the existence of close interaction between animals (cats and dogs) and humans.

Educating the pet owners as well as public mainly focusing on the prevention of zoonotic diseases should include the importance of personal hygiene, cleaning up animals faeces, proper and regular disposal to reduce environmental contamination as well as minimizing exposure of children to potentially contaminated environments (Chomel, 2008).

As cats and dogs are reservoirs for many zoonotic diseases to human as well as responsible for environmental contamination, antihelminthic treatment is most effective, if integrated early and targeted to these animal populations (Chomel, 2008). Various guidelines for the treatment and control of parasitic infections in carnivorous pets have been proposed in the USA (Centers for Disease Control and Prevention, and the Companion Animal Parasitology Council) and in Europe (European Scientific Counsel Companion Animal Parasitology) (Chomel, 2008). Moreover, alternative ways for preventing zoonotic diseases include the use of protective footwear in the parks, playgrounds or beaches, washing hands before handling food or eating, population control of dogs and cats, preventing animals from entering the public places, strict rule for animals free zone especially in recreational parks and playgrounds. Furthermore, the involvement of international agencies and institutions, such as the World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the International Livestock Research Institute, as well as the commitment of policymakers, scientists and field workers, are key means for the sustainable control and prevention of parasitic zoonoses (Chomel, 2008; Guillot & Bouree, 2007). Hence, prevention and control of parasitic zoonoses require global commitment from not only the different health professionals but also politicians and economists to allocate appropriate funding in order to conduct interactive control programs (Chomel, 2008).

5.2 Limitations of the study

The limitations of the study involved targeting newly arrived stray cats and dogs every week for faecal samples collection in animal shelters‘ houses (PAWS and SPCA) in order to make sure no repetition from the same cage. Moreover, extra care was needed when collecting samples from the dogs‘ cage. However, the researcher received generous help and assistance from the animal shelters‘ personnels during faecal samples collection. Besides, the result in this study was based on a single collection of the faecal samples, and this may result in a lower prevalence than the actual parasitic infection among these cats and dogs.

CHAPTER 6: CONCLUSION

Our results provide important information regarding the prevalence of intestinal parasites present in free-roaming stray animals and environmental soil samples in Klang Valley in the central-west region of Malaysia. Most of the parasites found in the current study are zoonotic parasites that are potentially capable of infecting human hosts. Moreover, *Spirometra* species, which was previously absent among stray cats, were reported in this study. This could be due to the characteristic nature of cats which tend to eat infected paratenic hosts from the surroundings.

Most importantly, the current study has drawn attention to the fact that *A. ceylanicum* is the most dominant species of hookworm, not only in stray cats and dogs, but also in the environmental soil of Klang Valley, Malaysia. This indicates that stray cats and dogs can be held responsible for zoonotic hookworm infections as well as environmental pollution. Additionally, *A.caninum*, which was previously known to be the dog hookworm, were also found in the cat. This suggested that cats are now the victims of dog hookworms due to the coexistence nature of the cats and dogs in the communities.

Nonetheless, the complementary approach to hookworm control and other zoonotic parasites of cats and dogs may be achieved by preventive measures, such as preventing cats and dogs from defecating in public areas, cleaning up animal wastes to

reduce parasitological contamination in the environment and educating the public to use protective footwear especially in the parks and playgrounds.

6.1 Suggestions for further studies

Further studies on humans especially those living in close proximity with stray animals and/or those involved in soil related activities should be included in the future studies. Although the current study further identifies the hookworms into species level, it is also important to carry out further research on species identification of other intestinal parasites such as *Ascaris* spp, *Trichuris* spp, and *Toxocara* spp in order to identify the original host(s) of these helminths.

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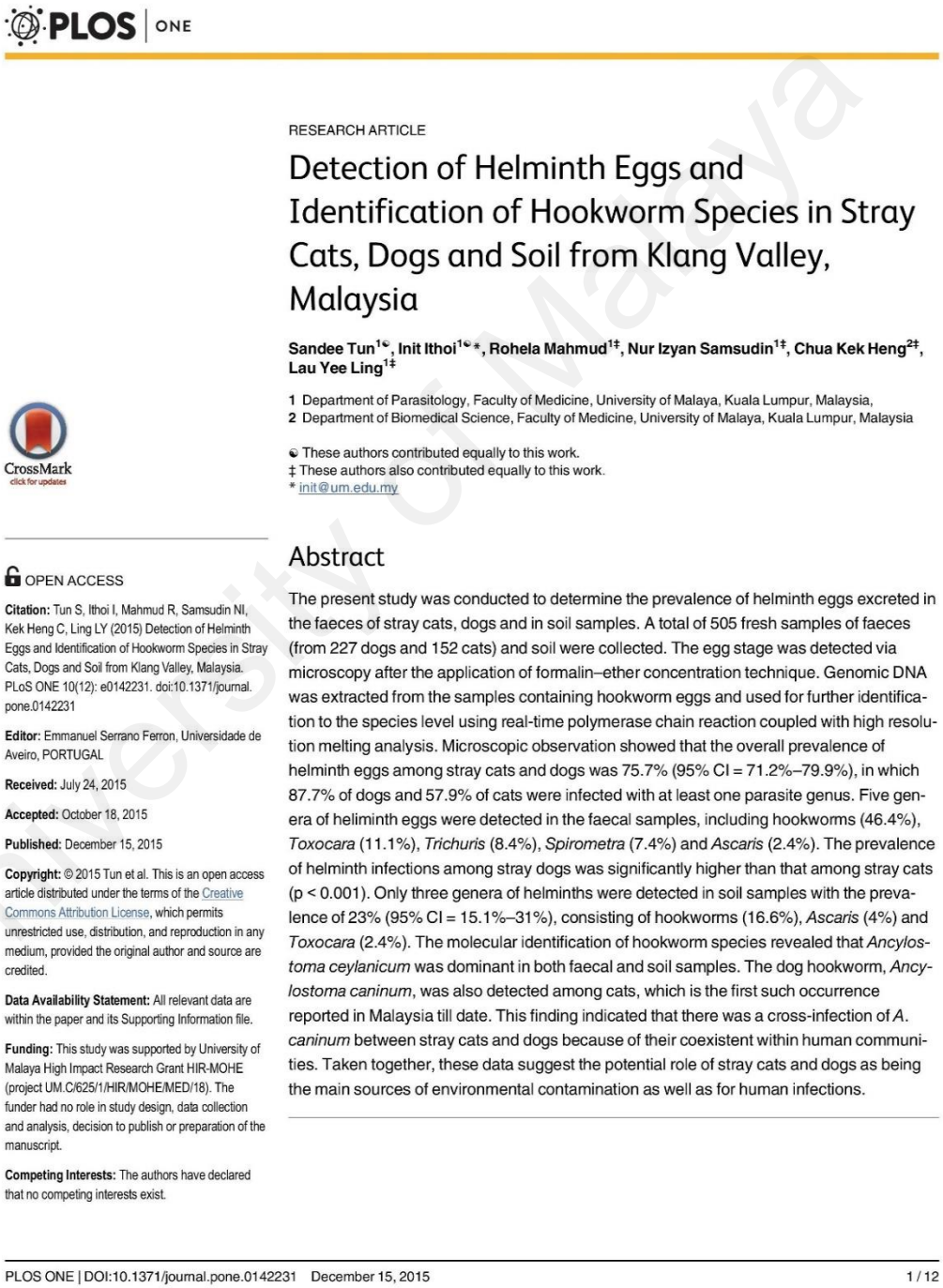
LIST OF PUBLICATIONS AND PAPERS PRESENTED

Journal

1. **Sandee Tun**, Init Ithoi, ohela Mahmud, Nur I yan Samsudin, Chua Kek Heng and Lau Yee Ling. Detection of helminth eggs and identification of hookworm species in stray cats, dogs and soil from Klang Valley, Malaysia. **Plos One**, 2015. 10(12): p. e0142231.



PLOS | ONE

RESEARCH ARTICLE

Detection of Helminth Eggs and Identification of Hookworm Species in Stray Cats, Dogs and Soil from Klang Valley, Malaysia

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Abstract

The present study was conducted to determine the prevalence of helminth eggs excreted in the faeces of stray cats, dogs and in soil samples. A total of 505 fresh samples of faeces (from 227 dogs and 152 cats) and soil were collected. The egg stage was detected via microscopy after the application of formalin–ether concentration technique. Genomic DNA was extracted from the samples containing hookworm eggs and used for further identification to the species level using real-time polymerase chain reaction coupled with high resolution melting analysis. Microscopic observation showed that the overall prevalence of helminth eggs among stray cats and dogs was 75.7% (95% CI = 71.2%–79.9%), in which 87.7% of dogs and 57.9% of cats were infected with at least one parasite genus. Five genera of heliminth eggs were detected in the faecal samples, including hookworms (46.4%), *Toxocara* (11.1%), *Trichuris* (8.4%), *Spirometra* (7.4%) and *Ascaris* (2.4%). The prevalence of helminth infections among stray dogs was significantly higher than that among stray cats ($p < 0.001$). Only three genera of helminths were detected in soil samples with the prevalence of 23% (95% CI = 15.1%–31%), consisting of hookworms (16.6%), *Ascaris* (4%) and *Toxocara* (2.4%). The molecular identification of hookworm species revealed that *Ancylostoma ceylanicum* was dominant in both faecal and soil samples. The dog hookworm, *Ancylostoma caninum*, was also detected among cats, which is the first such occurrence reported in Malaysia till date. This finding indicated that there was a cross-infection of *A. caninum* between stray cats and dogs because of their coexistent within human communities. Taken together, these data suggest the potential role of stray cats and dogs as being the main sources of environmental contamination as well as for human infections.

OPEN ACCESS

Citation: Tun S, Ithoi I, Mahmud R, Samsudin NI, Kek Heng C, Ling LY (2015) Detection of Helminth Eggs and Identification of Hookworm Species in Stray Cats, Dogs and Soil from Klang Valley, Malaysia. PLoS ONE 10(12): e0142231. doi:10.1371/journal.pone.0142231

Editor: Emmanuel Serrano Ferron, Universidade de Aveiro, PORTUGAL

Received: July 24, 2015

Accepted: October 18, 2015

Published: December 15, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information file.

Funding: This study was supported by University of Malaya High Impact Research Grant HIR-MOHE (project UM.C/625/1/HIR/MOHE/MED/18). The funder had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Manuscript prepared for publication

1. Epidemiology of intestinal parasites in faecal samples from stray cats, dogs and soil contamination in rural areas among Orang Asli Settlement in Malaysia

Conference attended for students' poster presentation

51st Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine (MSPTM) –Tropical Diseases in Malaysia: Innovative Approaches for Emerging Issues"

Poster Title:

SPECIES IDENTIFICATION OF HOOKWORMS IN STRAY CATS AND DOGS IN SELANGOR USING HIGH RESOLUTION MELTING (HRM) ANALYSIS

3rd – 4th March 2015, Kuala Lumpur, Malaysia

APPENDICES

APPENDIX A 1: Formalin-ether concentration technique (Faecal samples)

Approximately 1.5 gram of faecal sample was mixed with 7ml of 10% formalin (27.0 ml of 37% formaldehyde in 73 ml of 0.85% sodium chloride) in a clean plastic centrifuge tube

↓

Mixture was stirred using a wooden stick to form a suspension and was filtered through wet gauze into a clean 15 ml test tube; if necessary, adjusted to a total volume of 7 ml by topping up with 10% formal saline

↓

3 ml of ether was added into the supernatant and the mixture was vigorously mixed for 1 to 2 min and centrifuged for 5 min at 2,000 rpm

↓

The centrifugation resulted in 4 layers: The ether layer, the debris plug, the formalin layer and the sediment with parasites at the bottom

↓

The debris plug was freed from the sides of the tube with an applicator stick and the supernatant was decanted by inverting the centrifuge tube in one smooth motion

↓

The sediment was withdrawn with a Pasteur pipette and mixed with 0.85% normal saline or iodine on a clean dry microscopic slide

↓

The smear was covered with a coverslip

↓

Slide was examined thoroughly using light microscope under a low power (10x magnification) and high power (40x magnification) for detection of intestinal parasites

APPENDIX A 2: Formalin-ether concentration technique (Soil samples)

Approximately 10 gram of the soil sample was mixed with 14 ml of 10% formalin (27.0 ml of 37% formaldehyde in 73 ml of 0.85% sodium chloride) in a clean container cup

↓

Mixture was stirred using a wooden stick to form a suspension and was filtered through wet gauze into a clean dry plastic centrifuge tube

↓

The above step was repeated three times (10 g of soil and 14 ml of 10% formalin were used each time) to increase the amount of filtered material

↓

The supernatant was removed and top up the mixture with 10% formal saline adjusting a total volume to 14 ml

↓

6 ml of ether was added into the suspension and the mixture was vigorously mixed for 1 to 2 min and centrifuged for 5 min at 2,000 rpm

↓

The centrifugation resulted in 4 layers: The ether layer, the debris plug, the formalin layer and the sediment with parasites at the bottom

↓

The debris plug was freed from the sides of the tube with an applicator stick and the supernatant was decanted by inverting the centrifuge tube in one smooth motion

↓

The sediment was withdrawn with a Pasteur pipette and mixed with 0.85% normal saline or iodine on a clean dry microscopic slide

↓

The smear was covered with a coverslip

↓

Slide was examined thoroughly using light microscope under a low power (10x magnification) and high power (40x magnification) for detection of parasites

APPENDIX B: Trichrome staining technique

Faecal smear was prepared from fresh specimen or 2.5% potassium dichromate-preserved faecal sediment obtained from concentration technique and allowed to dry overnight

The smear was fixed in 70% ethanol (70 ml of 100% ethanol in 30 ml distilled water) for 5 minutes

Slide was then placed in 70% acid ethanol (0.5 ml of glacial acetic acid in 99.5ml of 70% ethanol) for 1 to 3 seconds

Slide was dipped several times in 95% ethanol followed by two separate 95% ethanol (5 ml distilled water in 95 ml of 100% ethanol) for 3 minutes each

It was washed two times with xylene for 5 to 10 minutes each

Slide was mounted with DPX and covered with a coverslip

Slide was allowed to dry overnight at room temperature

Slide was examined using light microscope starting from low to high magnification and oil immersion (10x, 40x, 100x)

APPENDIX C: Ziehl-Neelsen Staining Technique

Faecal smear was prepared from fresh faecal specimen on a clean glass slide and allowed to air dry

The smear was fixed with absolute methanol for 5 minutes

The smear was flooded with carbol fuchsin stain and was left for 8 to 10 minutes

The smear was then washed in tap water and drained

The smear was differentiated in 3% acid alcohol (3ml of 3% HCl in 97ml of 95% ethanol) until colour ceased to flood out. Smear should be very pale in colour

The smear was rinsed briefly in tap water

Next, the smear was counter stained with 0.5% malachite green for 30 seconds followed by rinsing in tap water and blotted dry

Slide was mounted with DPX and covered with a coverslip

The smear was covered with oil immersion and examined under 100x objective

APPENDIX D: Photograph scenes during sample collection

i. Stray cats from PAWs and SPCA shelters

ii. Stray dogs from PAWs and SPCA shelters

iii. Collection sites for soil samples in recreational parks