MULTIPLEX REAL-TIME PCR FOR THE DETECTION OF PATHOGENIC INTESTINAL PARASITES AND COMPARISON WITH PARASITOLOGICAL TECHNIQUES

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by

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LIST OF ABBREVIATIONS

	Description	Abbreviations
1	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside	X-gal
2	Acquired Immune Deficiency Syndrome	AIDS
3	Base pair	bp
4	Basic Local Alignment Search Tool	BLAST
5	Black hole quencher	BHQ
6	Bovine serum albumin	BSA
7	Calcium chloride	$CaCl_2$
8	Calcium chloride dehydrate	$CaCl_2.2H_2O$
9	Celcius	C
10	Centers for Disease Control and Prevention	CDC
11	Centimeter	cm
12	Complementary DNA	cDNA
13	Cycle threshold	Ct
14	Deoxyribonucleic acid	DNA
15	Direct immunofluorescence assay	DFA
16	Disodium hydrogen phosphate	Na_2HPO_4
17	Double-stranded DNA	dsDNA
18	Eggs per gram	EPG
19	Enzyme-linked immunosorbent assay	ELISA
20	Ethidium bromide	EtBr
21	Ethylenediaminetetraacetic acid	EDTA
22	Fluorescence resonance energy transfer	FRET
23	Full examination microscopic examination	FEME
24	High-performance liquid chromatography	HPLC
25	Hydrochloric acid	HCl
26	Immunofluorescence assay	IFA
27	Indirect hemagglutination assay	IHA
28	Internal transcribed spacer	ITS
29	Iodine	I_2
30	Larvae stage 1	L1
31	Larvae stage 2	L2
32	Larvae stage 3	L3
33	Larvae stage 4	L4
34	Loop-mediated isothermal DNA amplification	LAMP
35	Magnesium chloride	$MgCl_2$
36	Microliter	µl
37	Micrometer	µm
38	Micromolar	µM
39	Mililiter	ml/mL
40	Milligram	mg
41	Millimeter	mm
42	Milimolar	mM
43	Minor groove binder	MGB

44	Multiplex tandem PCR	MT-PCR
45	Nanometer	nm
46	Nanomolar	nM
47	National Center for Biotechnology Information	NCBI
48	Non fluorescent quencher	NFQ
49	Non template control	NTC
50	Number	No.
51	Optical density	OD
52	Phosphate buffered saline	PBS
53	Plaque forming unit	PFU
54	Polyvinylpyrrolidone	PVPP
55	Polymerase chain reaction	PCR
56	Potassium dihydrogen phosphate	KH_2PO_4
57	Potassium iodide	KI
58	Quantitative polymerase chain reaction	qPCR
59	Random amplification of polymorphic DNA	RAPD
60	Restriction fragment length polymorphism	RFLP
61	Revolutions per minute	rpm
62	Ribonucleic acid	RNA
63	Ribosomal DNA	rDNA
64	Single-stranded DNA	ssDNA
65	Small subunit ribosomal RNA	SSUrRNA
66	Sodium acetate	NaOAc
67	Sodium chloride	NaCl
68	Soil-transmitted helminth	STH
69	Specific gravity	SG
70	Synergy Brands, Inc.	SYBR
71	Tetramethylrhodamine	TAMRA
72	Tris-borate-EDTA	TBE
73	Ultra violet	UV
74	Unit	U
75	Weight per volume	w/v
76	World Health Organization	WHO
77	Zinc sulphate	$ZnSO_4$
78	Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$

PENGESANAN PARASIT PATOGENIK USUS MELALUI KAEDAH 'REAL -TIME MULTIPLEKS PCR' DAN PERBANDINGAN DENGAN TEKNIK-TEKNIK PARASITOLOGI

ABSTRAK

Jangkitan parasit dalam usus oleh helmin (cacing) dan protozoa adalah antara jangkitan yang paling biasa berlaku dan kekal sebagai masalah besar di kalangan masyarakat awam terutamanya di negara membangun. Jangkitan parasit usus yang berkaitan dengan helmin biasanya menyebabkan kesakitan di bahagian abdomen dan disertai oleh anereksia, loya dan cirit-birit, manakala protozoa usus lazimnya menyebabkan cirit-birit.

Kaedah pengesanan rutin yang dijalankan untuk mengesan jangkitan parasit usus biasanya bergantung kepada pengesanan dengan mikroskop. Bagaimanapun, kaedah ini tidak sensitif dan memerlukan kepakaran mikroskopi untuk mengelakkan 'misdiagnosis' mahupun 'overdiagnosis'. Kekurangan ini telah mencetuskan pembangunan kaedah yang lebih sensitif menggunakan DNA. PCR adalah satu kaedah yang terbukti sensitif dan spesifik untuk mengesan kehadiran organism yang patogenik di dalam usus. Memandangkan PCR secara konvensional mengambil masa panjang serta mudah terdedah kepada pencemaran, maka pembangunan kaedah pengesanan 'real-time PCR' yang pantas dan mampu memberikan data secara langsung dan kuantitatif ke atas produk PCR adalah bermanfaat.

Dalam kajian ini, satu kaedah 'real-time multipleks PCR' untuk mengesan spesies helmin usus yang berkaitan rapat iaitu *Ascaris lumbricoides*, *Strongyloides*

stercoralis, *Necator americanus* dan *Ancylostoma duodenale* dan satu kaedah 'real-time multipleks PCR' untuk mengesan protozoa usus yang juga berkaitan rapat iaitu *Entamoeba histolytica, Cryptosporidium parvum* dan *Giardia lamblia* telah dioptimumkan dan dinilai. Keputusan yang diperolehi telah dibandingkan dengan keputusan yang didapati menggunakan teknik parasitologi.

Sebanyak 302 sampel tinja telah dipungut daripada pesakit yang mengalami masalah gastrousus daripada Hospital Serian, Hospital Lundu dan Hospital Universiti Sains Malaysia. Teknik parasitologi yang dilakukan ke atas sampel tersebut adalah kaedah smir langsung, pengapungan / pemendapan dengan larutan zink sulfat, pewarnaan trikrom untuk *E. histolytica* dan *G. lamblia*, pewarnaan 'acid fast' untuk *C. parvum* dan kaedah Kato-Katz untuk mengesan ova helmin.

Primer yang digunakan dalam kajian ini telah diuji menggunakan lima tahap kepekatan primer dan dua profil suhu PCR. Produk PCR diklon ke dalam vektor pengklonan TOPO® dan plasmid DNA yang diperolehi disimpan sebagai kawalan positif untuk pembinaan lengkung piawai ('standard curve'). Daripada lengkung piawai yang diperolehi, had pengesanan bagi setiap organisma adalah seperti berikut: satu salinan DNA untuk *G. lamblia*, 10 salinan DNA untuk *Ancylostoma, A. lumbrocoides* dan *S. stercoralis*, dan 10^2 salinan DNA untuk *N. americanus, E. histolytica* dan *C. parvum.* Untuk kaedah 'real-time multipleks PCR', pengoptimuman had kepekatan primer juga dilakukan untuk mengelakkan perencatan kaedah PCR yang mungkin berlaku disebabkan oleh templat DNA yang mempunyai ketumpatan tinggi. Phocine herpesvirus 1(PhHV-1) dengan kepekatan yang optimum iaitu 10^{-2} PFU/ml telah dimasukkan bersama dalam setiap

pengekstrakan templat, ini bertujuan untuk mengesan sebarang keputusan negatif palsu yang mungkin disebabkan oleh kehadiran bahan perencat dalam sampel tinja ataupun disebabkan kegagalan proses PCR.

Setiap tindak balas yang dijalankan diuji dengan satu atau beberapa templat DNA dalam satu tiub, samaada dengan kehadiran DNA daripada PhHV-1 ataupun tanpa kehadiran DNA tersebut. Dua pengesanan menggunakan kaedah 'real-time multipleks PCR' dilakukan ke atas semua DNA, iaitu satu pengesanan untuk helmin dan pengesanan untuk protozoa. Keputusan yang diperolehi dibandingkan dengan keputusan teknik parasitologi berdasarkan ujian sensitiviti, spesifisiti dan Chi-square.

Berdasarkan keputusan daripada 302 sampel yang diuji, untuk kaedah pengesanan cacing, 13 (4.3%) sampel positif dikesan melalui kaedah mikroskopi manakala 94 (31.1%) sampel positif dikesan menggunakan kaedah 'real-time PCR'. Untuk pengesanan protozoa pula, lima (1.7%) sampel positif dikesan melalui kaedah mikroskopi manakala 23 (7.6%) sampel positif dikesan menggunakan kaedah 'real-time PCR' ($p < 0.05$). Jangkitan oleh dua atau lebih parasit usus (samaada helmin atau protozoa) dicatatkan dalam 29 daripada 110 sampel yang positif (26%) menggunakan kaedah 'real-time PCR' manakala kaedah mikroskopi tidak dapat mengesan kehadiran jangkitan berganda.

Kedua-dua kaedah 'real-time multipleks PCR' yang telah dioptimumkan dalam kajian ini telah berjaya mengesan semua organisma yang disasarkan. Kaedah-kaedah ini juga telah dinilai ke atas sampel pesakit dan berjaya mengesan empat jenis cacing usus dan tiga jenis protozoa usus yang penting. Untuk pengesanan cacing, kaedah

PCR berjaya mengesan 7.2 kali lebih banyak sampel positif manakala untuk pengesanan protozoa, kaedah PCR mengesan 4.6 kali lebih banyak sampel positif berbanding teknik parasitologi.

Kesimpulannya, kaedah ‘real-time PCR’ dalam kajian ini menyediakan satu kaedah alternatif untuk diagnosis parasit usus. Kaedah ini boleh diaplikasikan untuk diagnosis rutin pesakit, pemantauan rawatan dan kajian epidemiologi berkaitan jangkitan parasit usus.

MULTIPLEX REAL-TIME PCR FOR THE DETECTION OF PATHOGENIC INTESTINAL PARASITES AND COMPARISON WITH PARASITOLOGICAL TECHNIQUES

ABSTRACT

Intestinal parasitic infections by helminths and protozoa are among the most prevalent infections and remain a major public health burden in underdeveloped countries. Most intestinal helminth infections cause abdominal pain accompanied by aneroxia, nausea and diarrhea while intestinal protozoa cause diarrheal diseases.

Routine diagnostic methods for intestinal parasitic infections which rely heavily on microscopic detection are insensitive, and require well-trained microscopists to avoid misdiagnosis or overdiagnosis of the infection. These limitations have led to the development of highly sensitive DNA-based assays. PCR has been proven to be sensitive and specific for detection of enteric pathogens. Since conventional PCR is time consuming and prone to cross-contamination, it is desirable to develop a real-time PCR assay which is rapid and can provide quantitative and real-time information on the amplified products.

In this study, a real-time multiplex PCR assay for the detection of closely related intestinal helminths namely *Ascaris lumbricoides*, *Strongyloides stercoralis*, *Necator americanus* and *Ancylostoma duodenale* and an assay for the detection of three closely related intestinal protozoa which comprised *Entamoeba histolytica, Cryptosporidium parvum* and *Giardia lamblia* were optimized and evaluated.

A total of 302 stool samples were collected from patients with gastrointestinal problems from Hospital Serian, Hospital Lundu and Hospital Universiti Sains Malaysia. Parasitological techniques were performed by direct wet smear, zinc sulphate floatation/sedimentation, trichrome staining for *E. histolytica* and *G. lamblia*, acid-fast staining for *C. parvum* and Kato Katz technique for helminth ova.

The primers were tested at five concentrations and two PCR thermal profiles. The PCR products were cloned into TOPO® cloning vector and the DNA plasmids were used as positive control for standard curve construction. The detection limit for each organism was as follows: one DNA copy for *G. lamblia;* 10 copies for each *Ancylostoma, A. lumbrocoides* and *S. stercoralis*; and 10^2 copies for each *N. americanus, E. histolytica* and *C. parvum*. For real-time multiplex PCR assay optimizations, primer limitation steps were performed in order to avoid any inhibition due to high abundant template. Phocine herpesvirus 1 (PhHV-1) with an optimum dilution of 10^{-2} PFU/ml was included at the template preparation stage in order to detect false negative results due to the presence of any inhibitor compounds or PCR failure. Each assay was tested with one or multiple DNA template with or without the addition of PhHV-1 DNA in each reaction. Two real-time multiplex PCR assays i.e. for detection of intestinal helminths and intestinal protozoa were performed on all DNA samples. The real-time PCR results were compared with those obtained by parasitological techniques based on sensitivity, specificity and Chi-square tests.

For the detection of intestinal helminths, out of 302 samples, microscopic examination detected 13 (4.3%) positive samples while real-time PCR assay detected

94 (31.1%). For the detection of intestinal protozoa, microscopic examination detected five (1.7%) positive cases and real-time PCR detected 23 (7.6%) with $p<0.05$. Multiple infections by two and more organisms (either helminths or protozoa) were recorded in 29 out of 110 positive cases (26%) by real-time multiplex PCR, while no cases of multiple infections were reported by microscopic examination.

The real-time multiplex PCR assays optimized in this study successfully detected all the target organisms. They were also successfully evaluated on patients' samples for the detection of four important intestinal helminths and three common intestinal protozoa. The PCR assay detected 7.2 times more positive samples for intestinal helminths and 4.6 times for intestinal protozoa as compared to parasitological techniques.

In conclusion, the real-time PCR assays described in this study provide an alternative laboratory diagnostic method for intestinal parasitic infections and would be useful for treatment monitoring and epidemiological studies.

CHAPTER ONE

INTRODUCTION

1.1 Overview of parasitic infection

Parasitic infections are often caused by two most common type of organisms which are protozoa and helminths. Protozoa is single-celled organism meanwhile helminth is multicellular organism. Parasitic infection occurs when organisms live on or within a living host (usually mammals e.g. human). In general, parasite harms its host or survives by utilizing the host nutrients (*http://www.cdc.gov/parasites*).

Parasitic infections not only cause morbidity but also mortality in humans (Haque, 2007). Children and the elderly are highly susceptible to parasitic infections compared to other age groups due to their weakened immune systems and less capability to respond effectively to infections (Nagamani *et al.*, 2007). Immunocompromised or immunosuppressed people in cases of injury, surgery or chronic illness are also more vulnerable to acquire serious infections (Graczyk and Fried, 2007). In addition, children who are more exposed to soil when playing, have higher chances to be infected with soil-transmitted helminths. Parasitic infections are also responsible for many ill health conditions. The symptoms usually depend on the type of the parasite and the affected organ of the host. Moreover, prolonged or undetected infection may cause systemic problems which can affect the whole system of the human body. Most of the parasites undergo a complicated life cycle which includes the sexual and asexual types of reproduction within mammalian or invertebrate hosts.

1.2 Intestinal parasitic infections

Parasitic infections caused by intestinal helminths and protozoan parasites are among the most prevalent infections among humans in underdeveloped and developing countries (Haque, 2007). It is also among the leading causes of death and disease in tropical and subtropical regions of the world. Intestinal parasites refer to organisms that live in the intestine and consume the nutrition of the host. Common complications due to intestinal parasitic infections are abdominal discomfort, dysentery or mechanical irritation of intestinal mucosa and other general symptoms such as bloating, diarrhea and fever. Furthermore, when the burden of the infection is prominent, intestinal parasites may also cause serious health conditions and problems such as malnutrition, mental retardation and death especially in children (Amuta *et al.,* 2009).

1.2.1 Distribution and prevalence of intestinal parasitic infections

Intestinal parasites are distributed worldwide and many countries have high prevalence rates of the infection. It is estimated that there are more than three billion people infected with intestinal parasites throughout the world (Balcioglu *et al.,* 2007; Kurt *et al.,* 2007). Several factors affect the distribution and the prevalence of intestinal parasites. Personal hygiene, dietary habits, education level of the community, socio-economic status and climates are among the most common factors that influence the prevalence of intestinal parasitic infections (Balcioglu *et al.,* 2007; Mahsol *et al.,* 2008). Furthermore, overcrowded areas with inadequate hygiene and sanitation may increase transmission of parasitic infections. Therefore, the prevalence of intestinal parasitic infection is usually high in urban slum communities

where the socio-economic status is low, environmental sanitation and living condition is poor and water supply is unsafe with unhygienic personal habits (Noor Azian *et al.,* 2007).

1.3 Intestinal helminths

1.3.1 Overview

Intestinal helminths are macroparasites or multicellular organisms. Intestinal helminths include nematodes (roundworms), cestodes (tapeworms) and trematodes (flatworms) which live in the human gut. The common nematodes are the large roundworm *Ascaris lumbricoides,* the whipworm *Trichuris trichiura,* two species of blood-feeding hookworm *Necator americanus* and *Ancylostoma duodenale,* and the threadworm *Strongyloides stercoralis* (Chigozie *et al.,* 2007; Haque, 2007). They are known as geohelminths or soil-transmitted helminths (STHs).

STHs infect more than a billion people and among the most common cause of chronic infection to human (de Gruijter *et al.,* 2005; Wiria *et al.,* 2010). Recent estimates suggest that *A. lumbricoides* infects 1221 million people, *T. trichiura* infects 795 million, and hookworms infect 740 million people (de Silva *et al.*, 2003). Furthermore, infections with *S. stercoralis,* which affect 30 to 100 million people worldwide, are probably even more underestimated (Wiria *et al.,* 2010).

Commonly, infection with STH does not typically result in clinical disease. However, the effect of infections depends on several factors namely the helminth

species, the intensity of infection and the host immunological status. Majority of the infected individuals exhibit asymptomatic infections. This is because the pathology of STH is strongly related to the intensity of infection which is the number of worms, and usually, only a few worms are found in human body especially in early infection. The most common symptoms of intestinal helminth infection include diarrhea, foul breath, headache, nausea, abdominal pain and itching. Constipation and bloating can also arise, due to the intestinal organs obstruction (Baba *et al.,* 2009). Other symptoms associated with intestinal helminth infections are anaemia and asthma (da Silva *et al.,* 2008; Dori *et al.,* 2011).

1.3.2 General life cycle of soil-transmitted intestinal helminths

Intestinal helminth infection can be transmitted by fecal-oral route in the course of contact with parasite eggs or larvae in contaminated soil. Usually, infection occurs through accidental ingestion of *A. lumbricoides* or *T. trichiura* eggs or penetration of the skin by hookworm or *S. stercoralis* larvae. Intestinal helminths do not divide in the host (Haque, 2007) but they reproduce sexually. Male and female adult worms mate in the intestinal of the host and produce eggs. Adult worms inhabit in specific part of the host intestine; *A. lumbricoides*, hookworm and *S. stercoralis* live in the small intestine while *T. trichiura* inhabits the colon. Adult worms can produce large number of eggs and are able to survive for several years in the host, depending on the species. The female worms then discharge their eggs or larvae in human feces (Garg *et al.,* 2005).

Depending on the environmental conditions, the eggs (of *A. lumbricoides* and *T. trichiura*) can remain viable in the soil for several months while larvae (of hookworms and *S. stercoralis*) can remain viable for several weeks. Higher humidity of external environment is associated with faster development of the eggs to hatch into larvae. Some of the helminth larvae (namely *A. duodenale* and *S. stercoralis*) can undergo hypobiosis in the human body for several months. Hypobiosis is an arrested development at a specific point in the nematode life cycle. In this stage, the larvae do not grow and stop moving. They can survive for weeks or months before resuming development and may be resistant to some antihelminthics.

1.3.3 *Ascaris lumbricoides*

Ascaris lumbricoides is one of the most common and most widespread human infections, although *Ascaris suum* (the pig nematode) has also produced human infection. It is estimated that *A. lumbricoides* causes 1.2 million cases of acute illness and 10000 deaths annually. *A. lumbricoides* infection is rarely found in developed countries. However the infection rate is increased with travelling and migration in developing countries. The infection by *A. lumbricoides* is known as ascariasis. It occurs by ingestion of the infective *Ascaris* eggs which hatch into larvae in the small intestine. The larvae migrate to the caecum and proximal colon where they penetrate the mucosa. The larvae then move via the portal blood to reach the liver. After migration in the liver, the larvae advance to the lungs, penetrate the alveolar space and move to the pharynx where they are swallowed and return to the small intestine. Upon reaching the small intestine, they develop into adult worms, mate and produce eggs (Dold and Holland, 2011). The morphology of *A. lumbricoides* eggs in

diagnosis stage are shown in Figures 1.1.a - 1.1.b while the complete life cycle of *Ascaris* is further explained in Figure 1.1.c.

Infection of *A. lumbricoides* may be asymptomatic. Though, it may become life threatening as seen with hepatobiliary and intestinal obstructions due to heavy worm burdens (de Silva *et al.*, 1997; Crompton *et al.*, 1999). Hepatobiliary and intestinal obstruction are the main causes of morbidity and mortality due to ascariasis. Otherwise the infection may contribute to impaired nutrition, development, and educational progress especially in children (Hlaing, 1993).

In the stage when the larvae migrate to the pulmonary system, eosinophilic pneumonitis or Loeffler syndrome may develop. It happens when the eosinophils accumulate in the lung due to tissue destruction and releasing of the larval antigens. During this period, the patient may also develop asthma with hypersecretion of mucus, bronchiolar inflammation or has sputum containing eosinophils. In allergy-prone people, urticaria or itchiness of the skin may also occur. Infrequently, the larvae emerge through fistulae or fallopian tubes, urinary bladder, lungs or heart and pancreatic or bile duct (Noor Azian *et al.*, 2007; Varkey *et al.*, 2007). Women are believed to be more affected since the progesterone hormones play a role in inducing Oddi's sphincter relaxation which allows the nematode to access the biliary duct (Galzerano *et al.*, 2010). However, the most intense infections usually occur in children aged five to fifteen years due to higher exposure to contaminated environment and low immune system of the children. Treatment with albendazole was reported to give 93% reduction in egg count and a better cure rate (85% to 100%) than ivermectin treatment (78.4%) (Belizario *et al.*, 2003).

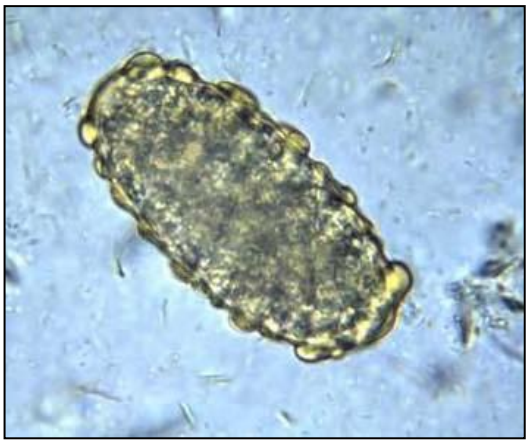


Figure 1.1.a *Ascaris lumbricoides* unfertilized egg. Elongated and covered by a visible mammillated layer.

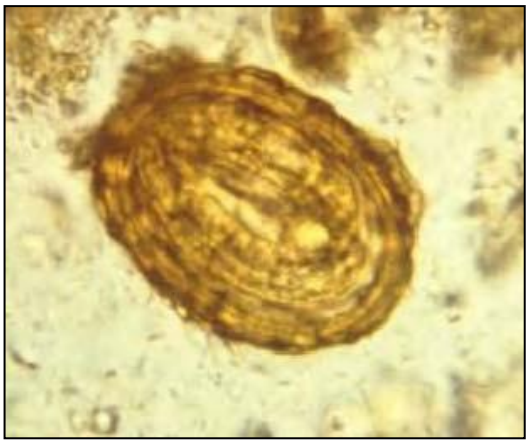


Figure 1.1.b *Ascaris lumbricoides* fertilized egg. Rounded, has a thick shell and smaller than unfertilized egg.

Source: http://www.phsource.us/PH/PARA/Diagnosing_Medical_Parasites.pdf

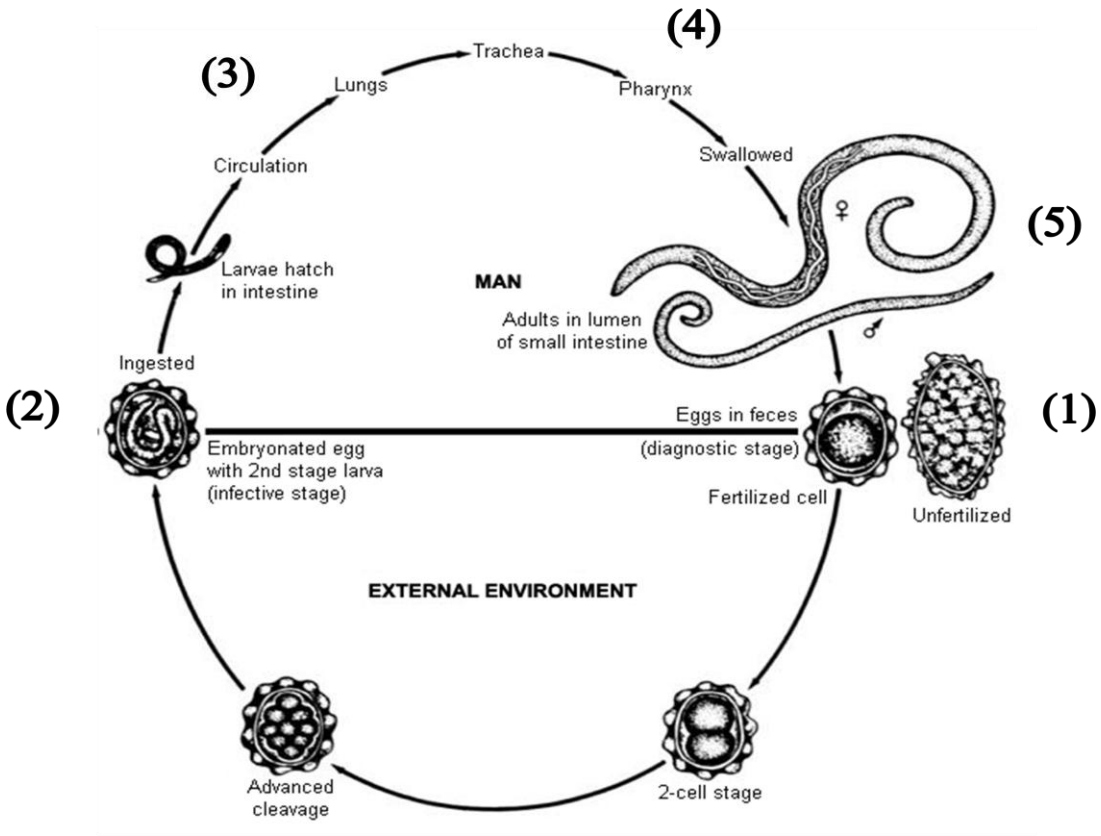


Figure 1.1.c *Ascaris lumbricoides* life cycle **1.** The life cycle begins with the production of 40 to 60 eggs by adult females living in the small intestine and deposited into external environment (soil) **2.** Eggs become infectious within several weeks and transmitted to the human host by ingestion or by inhalation of contaminated soil or dust **3.** Within the host, *Ascaris* larvae hatch in the jejunum, penetrate the intestinal wall, and migrate by hepatic venules to the right heart and pulmonary circulation **4.** They subsequently break through into alveolar spaces, ascend the trachea, and are swallowed back into the intestine **5.** They undergo a final moult and develop into adult worms (15 cm to 40 cm), which mate and generate a new generation of eggs.

Notes Under normal condition, the time from ingestion of eggs to development of new eggs is 10 to 12 weeks. Adult worms live for approximately one year before expelled from human body. Occasionally, male-only adult worms infection occur with yielding no eggs in stool while female-only adult worms infection produce infertile eggs, which never become infectious.

Source: https://online.epocrates.com/u/2924908/Ascariasis/Basics/Etiology

1.3.4 Hookworm

Primarily two species of hookworms infect humans; *Ancylostoma duodenale* and *Necator americanus*. The World Health Organization has catogerized *A. duodenale* and *N. americanus* as very destructive parasites (WHO, 2005). *N. americanus* tends to be prevalent in tropical climates, while *A. duodenale* is commonly found in a cooler and drier environment. However, their geographical distributions overlap considerably, and both species are endemic to many areas (Albonico *et al.,* 1998; Brooker *et al.,* 2004; Hotez *et al.,* 2005). Currently, it is estimated that 740 million people are infected worldwide, and more than 80 million of them are severely affected clinically (Bethony *et al.,* 2006).

Both species of the hookworm are transmitted through percutaneous route. The eggs are excreted in human feces. However, as opposed to *Ascaris*, hookworm eggs hatch into infective larvae in the feces or appropriate soil conditions. The infective larvae then infect humans by active invasion of the skin. They find and recognize their hosts by the behavioural phases of activation, directed crawling and penetration (Haas *et al.,* 2005). The infective, third-stage filariform larvae (L3) penetrate human skin and migrate via the circulatory system and the lung to finally reside as the adult stage (8 mm to 20 mm in length) usually in the duodenum. The adult worms can persist in the host within one to two years before being eliminated from the intestine (Gasser *et al.,* 2008).

Iron-deficiency anaemia caused by intestinal blood loss is the most outstanding feature of hookworm infection. Blood loss occurs when the adults worms attach via their buccal capsule to the small intestines, rupture capillaries and suck the blood

from the intestinal mucosa (Albonico *et al.,* 1998). The level of blood loss is proportionally related to the number of adult worms that inhabit in the host intestine. The risk and severity of iron-deficiency anaemia is described by the balance between iron intake and iron loss from intestinal bleeding. In heavy infections, hookworm can cause physical and mental retardation and deaths in children as well as adverse maternal-fetal outcomes in pregnant women (Hotez *et al.,* 2005).

A. duodenale is capable of oral infection through accidental ingestion of the eggs (Hoagland and Schad, 1978; Schad, 1991). Furthermore, *A. duodenale* larvae may undergo a dormant state (arrested stage of development in the musculature and/or intestine) after penetrating into the skin (Albonico *et al.*, 1998). Infection by *A. duodenale* may occur via transmammary routes; whereas *N. americanus* requires the transpulmonary migration routes (Setasuban *et al.,* 1980).

Currently, the control of hookworms has relied predominantly on antihelminthics drugs such as albendazole, mebendazole, pyrantel pamoate or levamisole (Bethony *et al.,* 2006). The morphology and the life cycle of hookworm are summarised in Figures 1.2.a, 1.2.b and 1.2.c.

Figure 1.2.a Hookworm larva. General morphology of both hookworm larvae is similar. Approximate length; 10 to 13µm for female and 8 to 11 µm for male worm.

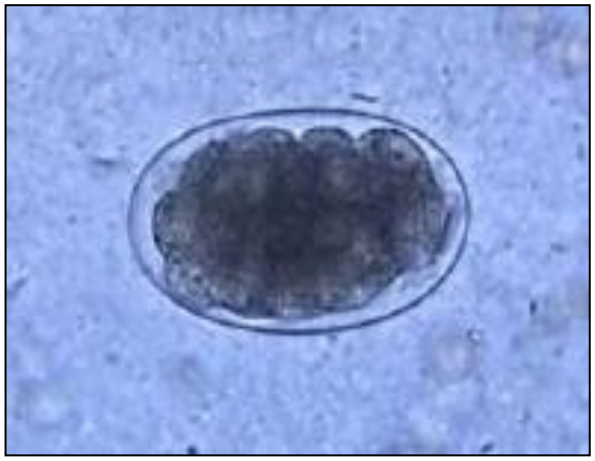


Figure 1.2.b Hookworm egg. General morphology of both hookworm eggs is similar. Oval shaped, 56 to 75 µm by 36 to 40 µm in size, with transparent and smooth thin shell.

Source: http://www.phsource.us/PH/PARA/Diagnosing_Medical_Parasites.pdf

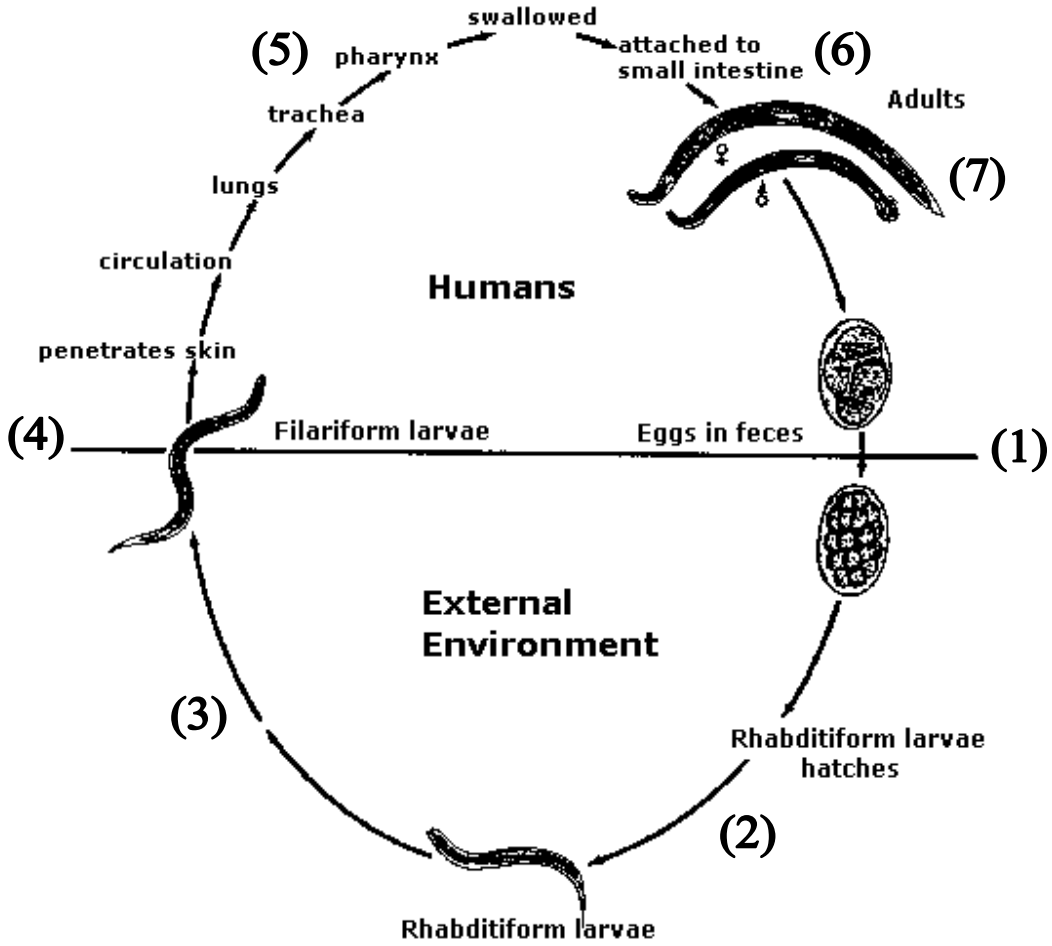


Figure 1.2.c Hookworm life cycle **1.** Hookworm eggs are passed in the stool. Under favourable condition; warmth, humidity and shade, the eggs develop into rhabditiform (L1) larvae **2.** Rhabditiform larvae hatch within one to two days **3.** The larvae grow and develop in the feces and/or the soil. After five to ten days (including two moults) they become filariform (L3) larvae which are infective to the human host. These ensheathed larvae can survive for three to four weeks under favourable environmental conditions **4.** Upon contact with the suitable host, the larvae penetrate through the skin of the host **5.** The larvae transported via the blood stream to the heart and then to the lungs. They migrate into the pulmonary alveoli, ascend the airways to the pharynx **6.** They are then swallowed and reach the small intestine and develop into mature adults. The adult worms attach to the small intestinal wall and feed on blood **7.** The worms copulate, and the females produce fertilized eggs, which are released in the feces.

Source: http://sprojects.mmi.mcgill.ca/tropmed/disease/intest-hookworm/life.htm

1.3.5 *Strongyloides stercoralis*

Strongyloides sp. can infect mammals, birds, reptiles and amphibians. To date, only two species of *Strongyloides* have been known to be able to infect humans; *Strongyloides stercoralis* and *Strongyloides fuelleborni*. However the most common and clinically important pathogenic species in humans is *S. stercoralis* whereas *S. fuelleborni* is found periodically in Africa and Papua New Guinea (Ashford *et al.*, 1992). *S. stercoralis* inhabits the small intestine of the host. Abdominal pain, nausea, vomiting, wheezing, and asthma are the common symptoms of *S. stercoralis* infection. During the infection, immunocompetent individuals may produce a moderate clinical symptom of diarrhea whereas it can be fatal in immunocompromised patients (Adenusi *et al.,* 2003; Olsen *et al.,* 2009; Feely *et al.,* 2010).

Strongyloides can undergo two types of development which are known as heterogenic development and homogenic development. In heterogonic development, *Strongyloides* eggs hatch in the feces and develop into L1 (first-stage larvae). L1 undergo L2, L3 and L4 stages and develop into rhabditiform male and female worms which are the free-living nematodes. These rhabditiform or the free-living nematodes will mate and the female lays eggs which hatch to release L1 which moult to an L2, then into infective filariform L3. These infective L3 are long lived and can persist in the environment until they come across a suitable host. This type of development is also known as indirect or sexual development. On the other way, homogenic development occurred when the L1 larvae is directly moult via L2 into infective L3 and persist in the environment until they encounter a suitable host. This type of development is also known as direct or asexual development (Viney and Lok., 2007).

Only the female worms of *Strongyloides* will be infective to the host. The female can either turn into L3 (infective) or free-living adult (non pathogenic) while the male worm can only develop into a free-living nematode. The infective larvae (L3) can penetrate into the skin host and migrate through the lungs until they are swallowed to reach the small intestine or else, they migrate in the host and moult via an L4 stage to develop adult parasitic female worms (Olsen *et al.*, 2009).

S. stercoralis also undergoes a dormant state as the same as *A. duodenale*. This dormancy of infective L3 larvae in human can cause chronic infection. At this stage, the infective L3 larvae can also cause transmammary transmission by re-activation of the larvae at lactation after their migration and arrestment in the mammary glands (Miller, 1981). Autoinfection can also occur in *Strongyloides* life cycle. Autoinfection is repeated generations of development in the same host individual. During autoinfection, the fast development of female larvae into infective L3 occurs within the gut and penetrates directly into the tissues of the primary host. Therefore, the whole life cycle is occurred within the host. During this time, infective L3 larvae may distribute through many organs and tissues of the host as well as the development of new female larvae in the gut (Vadlamudi *et al.*, 2006; Viney and Lok, 2007).

Strongyloides infections can be detected by the presence of *Strongyloides* eggs or larvae in the feces. Direct examination of thick smear on fresh fecal sample will reveal L1 stages and, or eggs. However, eggs are rarely found as they hatch in the intestine. High intensity of *Strongyloides* L1 can be detected by direct smear. Meanwhile, for lower intensity infections, larvae can be collected from feces using

concentration techniques. Fecal samples can also be grown to obtain the infective L3s. In severe and disseminated infection, sputum testing is also done to detect *Strongyloides* larvae (Smith *et al.,* 1985; Maayan *et al.,* 1987).

The drug of choice to treat *Strongyloides* infection is thiabendazole. However, ivermectin is also effective especially for disseminated infection (Datry *et al.,* 1994; Viney and Lok, 2007). The morphology and the life cycle of *S. stercoralis* are summarised in Figures 1.3.a, 1.3.b, 1.3.c, 1.3.d and 1.3.e.

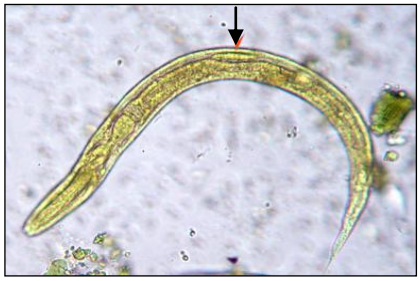


Figure 1.3.a *Strongyloides stercoralis* rhabditiform larva (L1) in iodine stained smear. Large genital primordium (arrow) with short buccal cavity, and sharp tail.

Figure 1.3.b *Strongyloides stercoralis* short buccal cavity in unstained wet mount of stool.

Figure 1.3.c *Strongyloides stercoralis* filariform larva (L3) characteristic; notched tail.

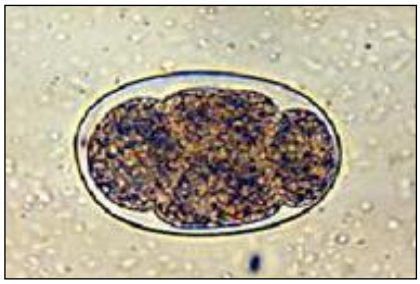


Figure 1.3.d *Strongyloides* egg. Oval and thin shelled, similar morphology with hookworm egg but smaller in size (50 to 58 by 30 to 34 µm). It is rarely found in stool as they hatch in the intestine.

Source: http://www.med.cmu.ac.th/dept/parasite/nematode/ssrlarva.htm
http://www.tropicalmed.eu/Page/WebObjects/PageTropE.woa/wa/displayPage?name=ReadingCultureAgarMicro

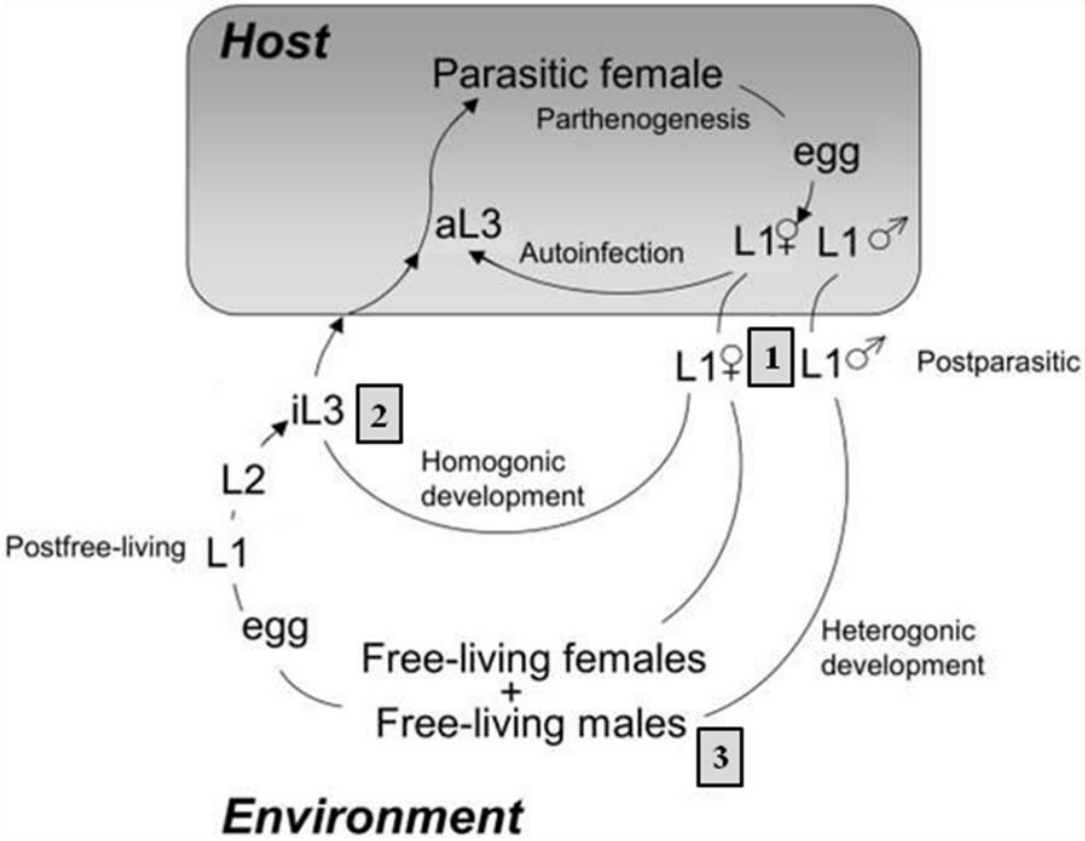


Figure 1.3.e *Strongyloides stercoralis* life cycle. The shaded box indicates the host. The figure shows three possible development of first stage larvae (L1) **1.** The autoinfective cycle, unique to *S. stercoralis*, in which development from the L1 to the autoinfective L3 (aL3) occurs within the gut of the primary host. Autoinfection can lead to explosive cycles of development and a highly pathogenic disseminated infection. All post-parasitic male L1 develop to free-living adult males. Post-parasitic female L1 passed in the feces may undergo development by either of two alternative pathways **2.** The homogonic cycle involves direct development to the infective L3 (iL3) **3.** Heterogonic development involves development to the free-living female and, following mating, production of a generation of free-living progeny. All progeny of the free-living adults develop to the iL3.

Source: http://www.ncbi.nlm.nih.gov/books/NBK19663/figure/A14969/?report=objectonly

1.4 Intestinal protozoa

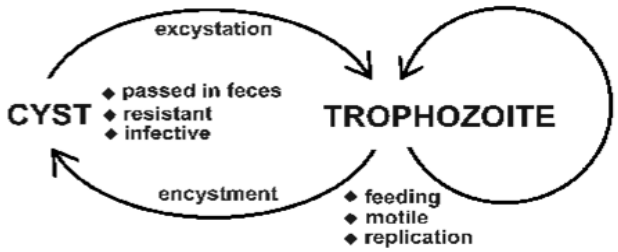
1.4.1 Overview

Protozoa are unicellular microparasites. Majority of the protozoa are non-pathogenic commensals. However, some of them can cause severe disease under certain circumstances. For example, *Giardia* (waterborne protozoa) produces mild diarrhea in immunocompetent individuals though in immunocompromised people, the infection can result in a severe disease. Intestinal protozoa are commonly found in tropical countries or areas with poor sanitary conditions. They are the more common causes of gastrointestinal infections in the developed countries as compared to helminths (Haque, 2007). Most intestinal protozoa has complex life cycle which enables the protozoa to adapt and replicate at high rates within the host (Garg *et al.,* 2005; Haque, 2007).

Entamoeba histolytica, Giardia lamblia, and *Cryptosporidium parvum* have been recognized as the three most important intestinal parasitic protozoa that cause diarrhea in human and other mammals (Marshall *et al.,* 1997; Garcia *et al.,* 2000; Guy *et al.,* 2004; Verweij *et al.,* 2004; Wang *et al.,* 2004; Coklin *et al.,* 2007; Haque, 2007). These protozoan parasites are acquired orally through contaminated food and water. In severe cases, they can produce chronic diarrhea which may lead to malabsorption, weight loss, and dehydration. The diseases they cause are known as amebiasis, giardiasis, and cryptosporidiosis respectively.

1.4.2 General life cycle of intestinal protozoa

A typical protozoan life cycle consists of cysts and trophozoites. Many intestinal protozoa exhibit a similar life cycle. After ingestion by the host, the cysts transform into trophozoites. Trophozoites is an active phase, in which protozoa are motile, acquires nutrient and undergoes asexual replication. If desiccation or low nutrients occurs, the trophozoites will secrete a thick wall that functions to protect them from dehydration and enter into a dormant period. The trophozoites develop into cysts instead of undergoing replication and is excreted with the feces, then ingested by the next host. The cysts will convert back into trophozoites when conditions are favourable.



Source: http://www.tulane.edu/~wiser/protozoology/notes/intes.html

1.4.3 *Entamoeba histolytica*

Amebiasis is a major cause of morbidity and mortality in the developing world (Furrows *et al.*, 2004). Currently, it is seen more frequently in developed country through the increase in travel (Tanyuksel *et al.*, 2005) and resulting in 100000 deaths per year (Verweij *et al.*, 2004). Several members of the genus *Entamoeba* infect humans. *E. histolytica* and *E. dispar* is morphologically similar and previously considered to be the same species but, genetic and biochemical data indicate that this

two species are distinct. However, *E. histolytica* or *E. dispar* can be distinguished from another species of *Entamoeba* by having peripheral chromatin in the nuclear and a smaller karyosome than *Entamoeba coli* and *Entamoeba hartmanni*.

Among various *Entamoeba* sp., only *E. histolytica* is considered pathogenic. *E. histolytica* can cause a severe intestinal disease characterized by amebic dysentery. Humans are the only host of *E. histolytica*. *E. histolytica* exhibits a typical fecal-oral life cycle which consists of infectious cysts that are passed in the feces and trophozoites that replicate within the large intestine. Infection occurs through the ingestion of cysts primarily by consuming contaminated food and water. Once the cysts ingested, excystation occurs to release the trophozoites. The trophozoites then secrete enzymes that digest the intestinal lining and leads to a perforated colon and peritonitis; an inflammation of the lining of the abdominal wall. Adherence of the trophozoites, cytotoxicity, and disruption of the tissues are important factors in the pathogenesis of *E. histolytica* (Ravdin, 1986; Petri *et al.*, 2002).

In severe cases, dehydration and anaemia may result from the loss of fluids and blood especially in children. Instead of infection in the intestinal tract of the host, the amebas can also metastasize to other organs such as liver and lungs and produce extraintestinal amebiasis. The liver is the most commonly affected organ and this is probably due to the direct transport of trophozoites from the large intestine to the liver via the hepatic portal vein and causing amebic liver abscesses. Haematogenous spread of trophozoites to other sites, such as the lungs, brain, spleen or pericardium

and cutaneous lesions can also occur, although it is extremely rare (Pelton *et al.,* 2006; Shenoy *et al.,* 2010; Maldonado *et al.,* 2011).

Only about 10% of the infected individuals will develop symptomatic invasive amebiasis and the remaining will undergo a non-invasive infection. The non-invasive infection is often asymptomatic but can cause diarrhea or other gastrointestinal symptoms (Tanyuksel *et al.,* 2005). This non-invasive infection can persist to an invasive amebiasis when the trophozoites invade the intestinal mucosa and kill the epithelial cells to produce ulcers and dysentery.

Previous studies show anti-ameba humoral responses in both asymptomatic and symptomatic *E. histolytica* infections (Sánchez *et al.,* 2002). Thus, this suggests that even in asymptomatic cases, there is a limited amount of invasion of the trophozoites. The symptom of infection depends on the organ infected. Hepatic infections are characterized by hepatomegaly, liver tenderness, and pain in the upper right quadrant, fever and anorexia while the symptoms of pulmonary amebiasis include cough, chest pain, dyspnea (difficult breathing) and fever. The sputum of the infected patient may be purulent or blood-stained and contain trophozoites (Wiser, 2011).

The best diagnosis of amebiasis requires the demonstration of *E. histolytica* cysts or trophozoites in feces or tissues (Fotedar *et al.,* 2007). Fresh stools can be immediately examined for motile trophozoites or preserved, stained and microscopically examined for cysts or trophozoites. Cysts will tend to predominate

in formed stools and trophozoites in diarrheic stools. Trophozoites with ingested erythrocytes are commonly found in the dysenteric feces (Wiser, 2011).

The extraintestinal infection is diagnosed by sigmoidoscopy for ulcers, especially in more severe disease (Madanagopalan *et al.,* 1968; Harries, 1982). Besides, the aspirates or biopsies can be examined microscopically for trophozoites that are most likely to be found at the abscess wall. Other methods such as antigen and antibody detection tests using enzyme-linked immunosorbent assay (ELISA), direct fluorescent antibody (DFA) or indirect haemagglutination (IHA) are available for detection of *E. histolytica* (Fotedar *et al.,* 2007).

The choice of drug for amebiasis depends on the clinical stage of the infection and the location of infection (lumen or tissue). The treatment is using antibiotics which kill the organism in the body, and followed or combined with an agent (lumenal anti-amebic drugs) which kills the parasite throughout the intestine. Several antibiotics are recommended for all symptomatic infections including metronidazole, tinidazole, tetracycline, and chloroquine (Kimura *et al.,* 2007). Iodoquinol, paromomycin or diloxanidefuroate are the luminal agents to treat asymptomatic cases (Petri and Singh, 1999; Wiser, 2011). In endemic areas where the rates of re-infection are high and treatments are expensive, only symptomatic cases would be treated. However, lumenal anti-amebic drugs are given to asymptomatic cyst passers to prevent the progression to severe disease and to control the spread of the disease (Wiser, 2011). The distribution of *E. histolytica* in human body, the morphology and the life cycle of *E. histolytica* are summarised in Figures 1.4.a, 1.4.b, 1.4.c and 1.4.d.

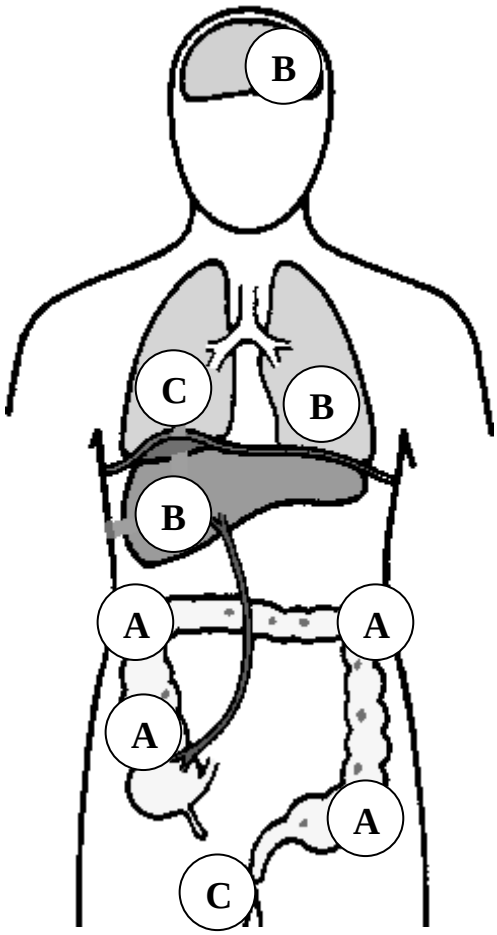


Figure 1.4.a Distribution of *Entamoeba histolytica* in human body **A.** *E. histolytica* is found primarily in the colon where it can live as a non-pathogenic commensal or invade the intestinal mucosa **B.** The ameba can metastasize to other organs via a blood stream; primarily involving the portal vein and liver **C.** The ameba can also spread via a direct expansion causing a pulmonary infection, cutaneous lesions or perianal ulcers.

Source: http://www.tulane.edu/~wiser/protozoology/notes/intes.html

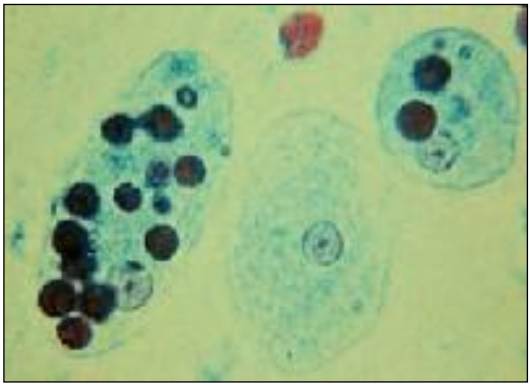


Figure 1.4.b *Entamoeba histolytica* trophozoites in trichrome stained smear. Two diagnostic characteristics are observed. Two trophozoites have ingested erythrocytes, and all three have nuclei with small, centrally located karyosomes.

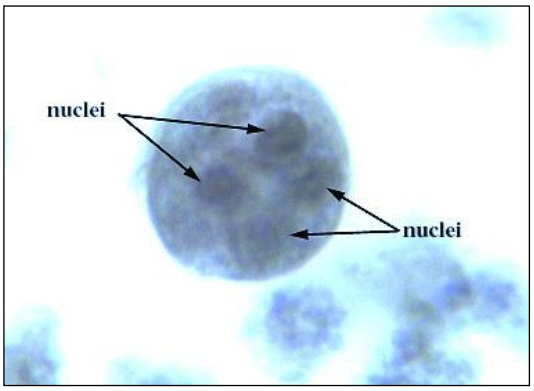


Figure 1.4.c *Entamoeba histolytica* cyst with four nuclei.

Source: http://umanitoba.ca/science/zoology/faculty/dick/z346/entahome.html

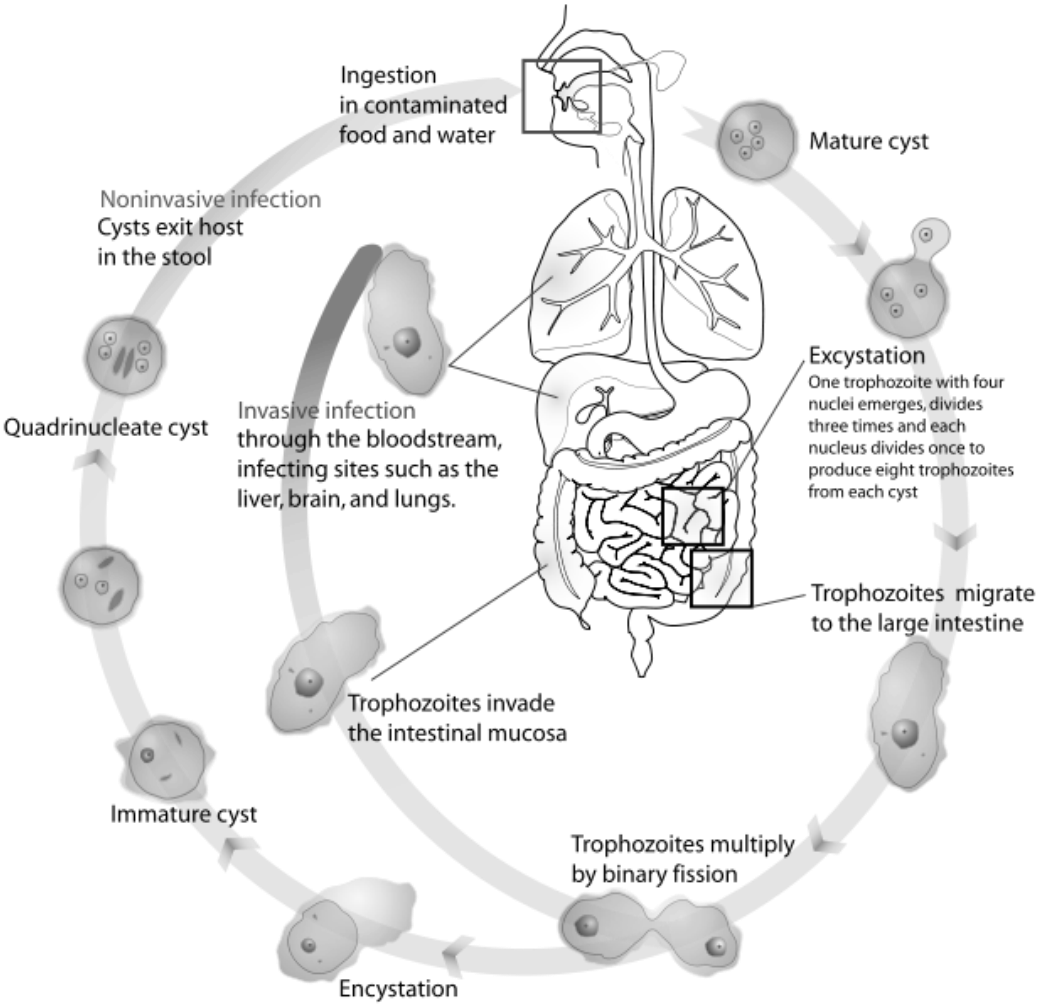


Figure 1.4.d *Entamoeba histolytica* life cycle **1.** Upon ingestion, the cyst passes into the small intestine and excystation occurs with transformation to the trophozoite stage **2.** The trophozoites migrate to the large intestine and colonized the host by asexual multiplication via binary fission. They can remain in the lumen or invade the wall of the intestine and multiply (for the pathogenic species). From here they can be transported via the circulation to other organs such as liver and lungs **3.** The cysts and trophozoites are passed in the feces of the infected host **4.** Infective stage of *E. histolytica* is the mature cyst **5.** The diagnostic stages are the trophozoite or cyst in stool or tissue specimens.

Source: http://commons.wikimedia.org/wiki/File:Entamoeba_histolytica_life_cycle-en.sgv