SERODIAGNOSIS OF STRONGYLOIDIASIS: IDENTIFICATION OF cDNA CLONES, PRODUCTION OF RECOMBINANT ANTIGENS AND IMMUNOASSAY DEVELOPMENT

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by

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LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-d-[3-ethylbenthiazoline sulfonate]
APS	ammonium persulfate
Au-GST	colloidal gold conjugate GST
Au-IgG4	colloidal gold conjugate IgG4
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
CAI	codon adaptation index
cDNA	complementary DNA
COV	cut-off value
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GST	glutathione s-transferase
His	histidine
HRP	horse-radish peroxidase
HTLV-1	Human T-cell lymphotropic virus
i.e.	id est (that is)
IFAT	immunofluorescence antibody test
INFORMM	Institute for Research in Molecular Medicine
IPTG	isopropyl-beta-D-thiogalactopyranoside
Kb	kilo base pair
kDa	kilo Dalton
LIPS	luciferase immunoprecipitation system
MALDI-TOF/TOF	matrix-assisted laser desorption/ionization-time of flight/ time of flight
MS	mass spectrometry
MW	molecular weight
NC	nitrocellulose membrane
Ni-NTA	nickel-nitrilotriacetic acid
OD	optical density
ORF	open reading frame
p*I*	isoelectric point
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
RC DC™	reducing agent detergent compatible
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
STH	soil-transmitted helminths
TB	terrific broth
TBS	tris buffered saline
TEMED	tetramethylethylenediamine
UV	ultra violet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

SERODIAGNOSIS UNTUK STRONGYLOIDIASIS:

PENGENALPASTIAN KLON cDNA, PENGHASILAN ANTIGEN

REKOMBINAN DAN PEMBANGUNAN IMUNOASAI

ABSTRAK

Strongyloidiasis merupakan penyakit parasit manusia yang disebabkan oleh cacing *Strongyloides stercoralis.* Jangkitan daripada cacing ini akan menyebabkan jangkitan jangka panjang dalam manusia, atau boleh disebarkan ke organ-organ lain, terutama bagi individu dengan sistem imun terkompromi, yang biasanya mengakibatkan kematian. Majoriti pesakit adalah tidak bersimptom, atau mengalami masalah gastrousus tak-spesifik, namun masih tiada kaedah 'gold standard' untuk mengesan jangkitan tersebut. Diagnosis definitif biasanya dilakukan melalui kombinasi tanda dan simptom klinikal, pengesanan mikroskopik, dan ujian serologi. Sehingga kini, ujian komersil yang sedia ada adalah berasaskan ekstrak asli antigen parasit, tetapi ujian tersebut mempunyai banyak kelemahan; contohnya masalah reaktiviti silang dengan jangkitan cacing yang lain. Ujian berasaskan antigen rekombinan merupakan alternatif yang sesuai bagi meningkatkan spesifisiti diagnostik dan keseragaman kualiti ujian, oleh itu, kajian ini dijalankan untuk mencapai matlamat ini. Peringkat awal kajian ini melibatkan pengujian sampel serum dengan *in-house* IgG-, IgG4- dan IgE-ELISA disamping kit komersial IgG-ELISA. Hasil keputusan mendapati bahawa asai IgG-ELISA menunjukkan sensitiviti yang tertinggi melalui kedua-dua asai *in-house* dan komersial (84.6%, n=26), manakala IgG4-ELISA menunjukkan spesifisiti yang tertinggi iaitu sebanyak 92.7% (n=55). Sampel serum dikategorikan mengikut kumpulan berdasarkan hasil keputusan IgG dan IgG4 untuk digunakan dalam eksperimen imunosaringan yang seterusnya.

Imunosaringan pertama perpustakaan faj cDNA *S. stercoralis* menghasilkan pemilihan sebanyak 20 cDNA klon bagi setiap faj imunoblot IgG- dan IgG4-. Klon-klon ini kemudiannya digunakan dalam imunosaringan kedua dan ketiga yang menggunakan sampel serum terjerap individu. Akhirnya, dua klon cDNA, iaitu Ss3a and SsIa, telah dipilih sebagai mempunyai nilai diagnostik yang berpotensi tinggi. Sisipan DNA bagi Ss3a adalah sangat serupa dengan protein hipotetikal *S. stercoralis*, manakala sisipan DNA bagi SsIa adalah sama dengan protein ikatan immunoglobulin *S. ratti*. Gen Ss3a telah diekspres secara rekombinan sebagai protein gabungan-GST di dalam vektor pET42a dan ia disahkan melalui analisis MALDI TOF/TOF. Nilai diagnostik bagi protein rekombinan rSs3a ditentukan melalui analisis western blot dengan menggunakan sampel serum individu. Seterusnya, ujian dipstik aliran sisi (1 jam) telah dihasilkan dengan sensitiviti dan spesifisiti sebanyak 100% (n=10) and 100% (n=10). Sementara itu, gen SsIa telah digabungkan dengan dua label protein dalam vektor pET28b dan diekspreskan sebagai protein gabungan-His, dan ia disahkan melalui analisis western blot dan MALDI-TOF/TOF. Kemudiannya, rSs1a telah dibangunkan kepada ujian pantas aliran sisi (15 minit), memberikan kadar sensitiviti sebanyak 90% (n=30) dan kadar spesifisiti sebanyak 98% (n=46). Kajian ini telah berjaya menemukan dua penanda jangkitan dan menghasilkan dua protein rekombinan dengan nilai diagnostik yang berpotensi. Bukti konsep penggunaan kedua-dua protein tersebut dalam ujian dipstik aliran sisi bagi serodiagnosis penyakit strongyloidiasis telah ditunjukkan, dengan kombinasi kedua-duanya mungkin sesuai digunakan sebagai ujian pantas untuk pengesanan penyakit strongyloidiasis pada manusia.

SERODIAGNOSIS OF STRONGYLOIDIASIS:

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ABSTRACT

Strongyloidiasis is a human parasitic disease caused by the nematode *Strongyloides stercoralis.* Infection by this parasite can cause long-term infection in humans or can disseminate to other organs, especially in individuals with immunosuppression, which commonly results in fatal outcomes. The majority of patients are asymptomatic or present with non-specific gastrointestinal complaints, and there is no gold standard method to rule out the infection. Definitive diagnosis is usually made by a combination of clinical signs and symptoms, microscopic identification, and serology test. To date, the available commercial tests are based on native parasite antigen extract, but such tests have problems of cross-reactivity with other helminthic infections. A recombinant antigen-based test is a good alternative for improved diagnostic specificity and standardized test quality, thus, the present study was conducted to achieve this goal. The initial stage of the study involved testing serum samples with the *in-house* IgG-, IgG4- and IgE-ELISAs in addition to a commercial IgG-ELISA kit. The results showed that the IgG-ELISA assay demonstrated the highest sensitivity using either *in-house* or commercial assays (84.6%, n=26), whereas the IgG4-ELISA displayed the highest specificity of 92.7% (n=55). The serum samples were categorized into different groups based on the IgG and IgG4 results for use in the subsequent immunoscreening experiments. Primary immunoscreening of the *S. stercoralis* phage cDNA library resulted in the selection

of 20 cDNA clones from each IgG- and IgG4-phage immunoblot. These clones were subjected to secondary and tertiary immunoscreenings using individual pre-adsorbed serum samples. Finally, two cDNA clones namely Ss3a and SsIa were selected as having high potential diagnostic value. The Ss3a DNA insert was highly similar to a *S. stercoralis* hypothetical protein, while the SsIa DNA insert was identical to an immunoglobulin-binding protein of *S. ratti*. The Ss3a gene was recombinantly expressed as a GST-fusion protein in the pET42a vector and validated by MALDI TOF/TOF analysis. The diagnostic value of rSs3a protein was determined by western blot analysis using individual serum samples. Subsequently, a one-hour lateral flow dipstick test was produced with diagnostic sensitivity and specificity of 100%, respectively. Meanwhile, the SsIa gene was fused to a dual protein tag in the pET28b vector and expressed as a His-fusion protein and verified by western blot analysis and MALDI-TOF TOF. The rSs1a was then developed into a rapid (15 min) lateral flow dipstick test, which gave a sensitivity rate of 90% (n=30) and a specificity rate of 98% (n=46). This study has successfully discovered two infection markers and produced two new recombinant proteins of potential diagnostic value. Proof-of-concept of their usefulness in a lateral flow dipstick test for serodiagnosis of strongyloidiasis was also demonstrated, and in combination may serve as a good rapid test for of human strongyloidiasis.

CHAPTER ONE

INTRODUCTION

1.1 Strongyloidiasis: The overview

Strongyloidiasis is a soil-transmitted helminthiasis capable of causing long-term infection as well as fatal consequences in humans. This disease is caused by two species of soil-transmitted helminths (STH) of the genus *Strongyloides,* namely, *Strongyloides stercoralis* and *S. fuelleborni,* with the former being the most prevalent species infecting humans. It is widely prevalent in populations living in tropical and sub-tropical climates, affecting approximately 100-370 million people worldwide (Bisoffi *et al*., 2013).

The World Health Organization (WHO) has listed 17 diseases as neglected tropical diseases (NTDs). Seven of them are parasitic diseases, namely, cysticercosis, dracunculiasis, echinococcosis, foodborne trematodiases, lymphatic filariasis, onchocerciasis, schistosomiasis, and soil-transmitted helminthiases (http://www.who.int/neglected_diseases/diseases/en/). These diseases were grouped as NTDs because they have been largely wiped out in the more developed parts of the world and persist only in the poorest, most marginalized communities and conflict areas; where they cause tremendous suffering due to their disfiguring, debilitating, and sometimes fatal impact (http://www.cdc.gov/globalhealth/ntd/).

Among the STH, attention has historically been focused on four species, namely, *Ascaris lumbricoides, Trichuris trichiura,* and hookworms *(Ancylostoma duodenale* and *Necator americanus)* (Krolewiecki *et al*., 2013). Infection by *S. stercoralis* has received less attention and often overlooked because of the major challenges presented in measuring the infection and the complex diagnostic methods.

Therefore, strongyloidiasis is mostly known as “the most neglected of the neglected tropical diseases” (Olsen *et al*., 2009).

Although the need for research to improve the diagnosis of strongyloidiasis had been highlighted over three decades ago, there is still no definitive or “gold standard” method to rule out the infection. This is the reason for the undetected persistence of the parasite inside the human body over decades, with the current record of chronic infection for 75 years (Prendki *et al*., 2011).

The clinical manifestations of the disease ranges from asymptomatic light infection to chronic symptomatic infection, or it may progress to life-threatening dissemination of larvae to all internal organs among individuals with compromised immune system (Olsen *et al*., 2009). It was reported that strongyloidiasis was accountable for a mortality rate of about 60-85% amongst immunocompromised patients (Ericsson *et al*., 2001) and contributed to 16.7% of the mortality rate for patients requiring hospitalization (Iriemenam *et al*., 2010).

Cases of patients infected with strongyloidiasis have recently increased due to the increasing numbers of transplant recipients and patients with altered immune status, malignancies and malnutrition. These complications are highly associated with the immunosuppression caused by corticosteroids and infection with human T lymphotropic virus type 1 (HTLV-1), the two most common risk factors for strongyloidiasis; however, the greatest risk is visiting an endemic area (Mirdha, 2010).

As strongyloidiasis contributes to serious outcomes in certain groups of populations, misdiagnosis and untreated infections could lead to fatality, prompting serious problems in the community. Therefore, understanding the nature, epidemiology, and immunological factors of *S. stercoralis* as well as the overall

aspect of strongyloidiasis may lead to better prevention, diagnosis, and treatment of the disease.

1.2 History of the discovery of *S. stercoralis*

S. stercoralis was first described back in July 1876 by Louis Normand, a military physician from France. He discovered a novel worm in the faeces of soldiers returning from World War II and the Cochin-China (Vietnam) war who were suffering from diarrhoea. The worm was then named *Anguillula stercoralis* by his colleague, Bavay, to reflect its shape (Latin: “anguillula” = “eel” and “stercus” = “dung”). Bavay was also the first to describe the free-living adults parasite he found *in vitro* (Grove, 1995).

Then, in 1878, Grassi and Parona from Italy reviewed the findings of this parasite and renamed it *Strongyloides intestinalis* (Greek: “Strongylos” = “round” and “Eidos” = “similar”). However, in 1881, the name of this parasite was once again changed by Perroncito, who called it *Pseudorhabditis stercoralis,* as he found and cultivated free-living adult worms from larvae identical to the *A. stercoralis*. Two years later, Leuckart realized that all of these worms were the same but from different phases in the life cycle of a single parasite. He then suggested the name “*Rhabdonema strongyloides”*. After several names had been suggested and changed, finally, in 1902, Stiles and Hassall pointed out that the correct name of this parasite should be *Strongyloides stercoralis,* and this name was accepted by the International Commission on Zoological Nomenclature in 1915 (Lindo and Lee, 2002).

The migratory route of this parasite inside the host was first studied by Fulleborn in 1914 (Cox, 2002). It was not until the 1930s, 50 years after its first

discovery that the full life cycle, pathology, and clinical features of *S. stercoralis* in humans were fully disclosed (Schär *et al*., 2013).

1.3 The organism *S. stercoralis*

1.3.1 Taxonomy

The species *S. stercoralis* is classified under the genus *Strongyloides,* family of Strongyloididae, order Rhabditida, class Secernentea, and phylum Nematoda. In the United States, this genus is commonly known as threadworm, and it is called pinworm in the United Kingdom (Liu, 2012). There are more than 52 species of the genus *Strongyloides;* however, only two species are known to parasitize humans, namely *S. stercoralis* and *S. fuelleborni,* with the former the most pathogenic species in humans and causes majority of the infections. Other than humans, dogs and primates are also susceptible to infection with *S. stercoralis* (Bonne-Année *et al.*, 2011).

S. fuelleborni is a zoonosis that usually occurs in non-human primates, and the main source of infection is monkey faeces. There are two subspecies of *S. fuelleborni,* namely *S.f. fuelleborni* and *S.f. kellyi,* which are reported to cause infection in humans in Africa and Papua New Guinea respectively (Cox, 2002). According to King and Mascie-Taylor (2004), *S.f. kellyi* is responsible for 27% of *Strongyloides* infections amongst children, with 81% of them being under 12 months old. It caused a potentially fatal disease among children called ‘swollen belly syndrome’ (SBS). The high percentage of incidence among young children might be due to the habits of the mothers, who carry their children in string bags lined with dried banana leaves and/or cloth. These materials are changed infrequently, and eggs and free-living larvae have been found (Brooker and Bundy, 2009).

The difficulty in maintaining *S. stercoralis* in the respective host limits the investigation into this organism; therefore, *S. ratti* and *S. venezuelensis*, which naturally infect rodents, have been used to study the biology and immunology of *Strongyloides* infection (Bonne-Année *et al.*, 2011). Some limitations have been encountered with such studies, as the results generated with rodent parasites do not mimic the actual pathogenesis of the human pathogen *S. stercoralis*. They migrate throughout the body; therefore the recovery of the parasites is problematic, and study of the microenvironment of the host is nearly impossible (Bonne-Année *et al.*, 2011).

1.3.2 Morphology

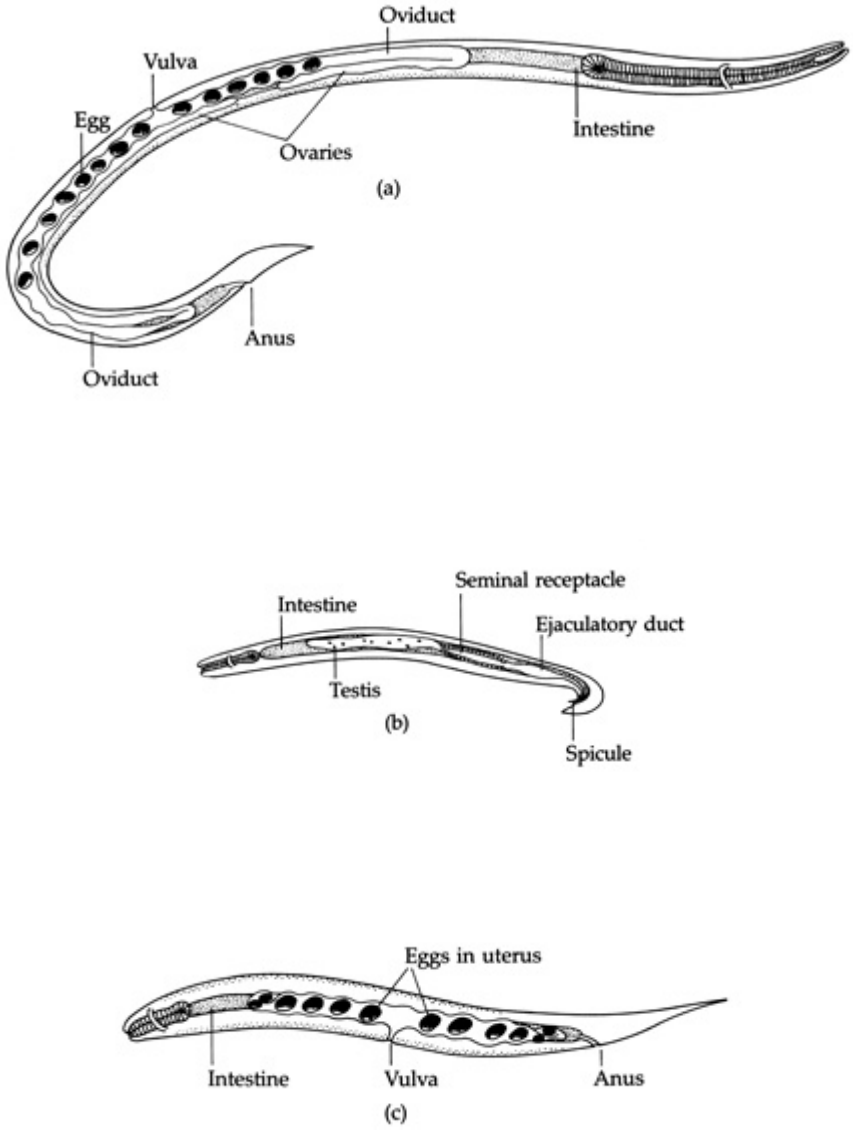
S. stercoralis has two different life cycles, namely, the parasitic and the free-living life cycles. The morphology of the parasite in the former is different from in the latter. Figure 1.1 shows the difference in the morphology of adult *S. stercoralis* larvae between the two life cycles, i.e. parasitic female, free-living male and free-living female.

The parasitic female of *S. stercoralis* is approximately 2.0-2.8 mm in length, has an average diameter of 37 µm, and a blunt-ended tail. It is very slender and threadlike, giving rise to the common name, 'threadworm' (Lindo and Lee, 2001). As described by Grove (1995), the body of the female worm is attenuated anteriorly, and it contains a long cylindrical oesophagus (also called the pharynx). The oesophagus is followed by the intestine and is surrounded by a nerve ring. The vulva is located in the mid-ventral line in the posterior third of the body (Grove, 1995). Parasitic worms are parthenogenetic females; their reproductive organs develop after the male reproductive organs have disappeared. There is no parasitic male (Simon, 2009).

The length of the free-living adult female is shorter, ranging from 0.9-1.7 mm, but the diameter is about 85 µm. It has a thin cuticle and is transparent, and the oesophagus is attached to the terminal mouth. The reproductive system is similar to that seen in the parasitic female except that each uterus contains numerous eggs (Grove, 1995). However, using a dissecting microscope, the free living adult female is easily seen in cultures, while the parasitic female is barely visible in stool samples, except in a very severe infection (Lindo and Lee, 2001). This is because the parasitic females lie embedded within the mucosal epithelium of the proximal small intestine where they deposit their eggs (Simon, 2009).

The free living male of *S. stercoralis* is approximately 1.0-1.2 mm long and 55 µm in diameter and has a typical J shape with a sharply pointed tail (Lindo and Lee, 2001). The mouth, oesophagus, and intestine are similar to those seen in the free-living female. The reproductive system consists of a blindly ending testis at the anterior end, and it is attached to a poorly demarcated vas deferens and seminal vesicle (Lindo and Lee, 2001).

The eggs of parasitic and free-living females are morphologically similar, with a thin-shelled, ellipsoidal shape, and measure between 40 x 70 µm in size (Grove, 1995). The eggs may be fully embryonated when laid or may have undergone several cell divisions (Lindo and Lee, 2001). The eggs hatch within the submucosa or during passage through the lumen of the intestine, liberating rhabditiform larvae, which are then excreted with the faeces (Nappi and Vass, 2002).



http://rowdy.msudenver.edu/~churchcy/BIO3270/Images/Nematodes/Strongyloides_stercoralis.htm

Figure 1.1 The morphology of a) parasitic female, b) free-living male, and c) free-living female of *S. stercoralis*.

1.3.3 Life cycle

The life cycle of *S. stercoralis* is unique, with its alternation between free-living and parasitic cycles enabling the worms to live in the environment and in the human host. Additionally, this parasite has the potential for autoinfection and multiplication within the host, making it different from any other nematode. Two types of life cycles exist, that is, an external sexual cycle involving free-living worms in the environment and an internal asexual cycle involving parasitic worms in the host. Therefore, reproduction takes place at two different sites. Figure 1.2 depicts a summary of its life cycle.

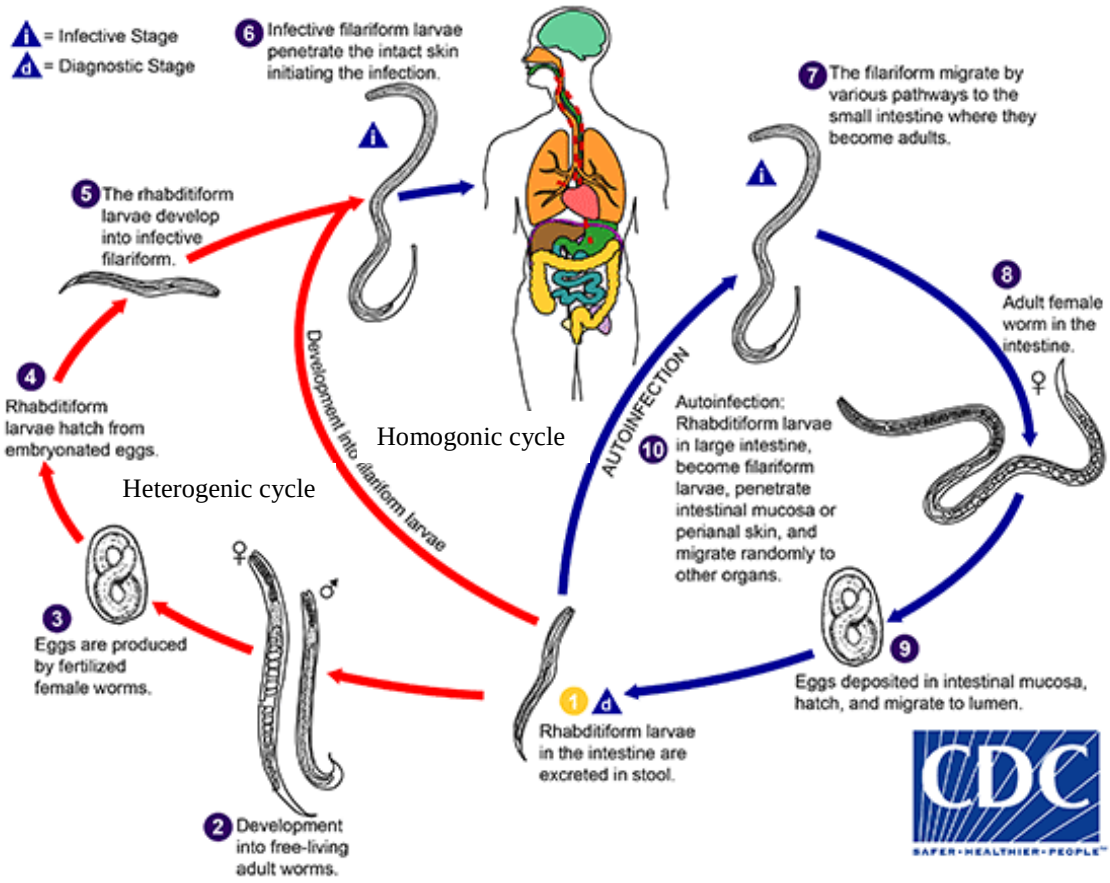
Infection is initiated when a free-living third-stage infective larva (L3) penetrate the skin of the human host. There is then a period of larval migration within the host, during which the L3 larvae undergo rapid development from the free-living third-stage larvae to a post-penetration, host-adapted transformed stage called the $L3^{+}$ (Bonne-Année *et al.,* 2011; Brigandi *et al*., 1998; Brigandi *et al*., 1997). The $L3^{+}$ differs antigenically as well as physiologically from the L3 due to the different environments where the two forms of larvae reside, as reported by Maruyama *et al.* (2006) (Bonne-Année *et al.,* 2011). Females are the only adult worms to form the parasitic stages of *Strongyloides,* as the male disappears from the bowel soon after oviposition (Viney and Lok, 2007).

Inside the host, eggs are produced by the female worms parthenogenetically, that is, via asexual reproduction without the presence of the adult male worm (Brooker and Bundy, 2009). The female worms deposit their eggs in the mucosal epithelium of the small intestine. The eggs then hatch to become rhabditiform larvae or are passed in the stool, and so enter the free-living cycle, which is sometimes called the external parasitic, indirect, or sexual cycle. Thus, the presence of

rhabditiform in the stool sample is considered as diagnostic of strongyloidiasis, since the eggs are rarely found (Lindo and Lee, 2001)

Inside the host's gut, the rhabditiform L1 emerge from the eggs and moult twice to become infective L3 larvae while they are still in the intestinal lumen of the host. The infective filariform larvae then penetrate either the intestinal mucosa, causing internal autoinfection, or the skin of the perianal area, causing external autoinfection (http://www.cdc.gov/parasites/strongyloides/biology.html). This cycle continues, with the female adult larvae producing eggs parthenogenetically 25-30 days after initially penetrating the skin (Liu, 2012). The ability of autoinfection to initiate a new life cycle allows the persistence of the infection for decades.

In the free-living stage, the rhabditiform larvae undergo further development in the soil, moulting directly to become infective filariform larvae via the homogonic cycle (Nappi and Vass, 2002). Alternatively, when the environmental conditions are optimal, rhabditiform larvae develop into free-living male and female adult worms that mate and produce eggs; the eggs hatch to release L1 larvae, moult again to become L2, and then again to become the infective filariform L3 stage through the heterogonic cycle (Grove, 1995). The infective L3 stages produced by these two cycles are long lived, and they can persist in the environment until they encounter a suitable host, continuing the life cycle following skin penetration (Viney and Lok, 2007).



http://www.cdc.gov/dpdx/strongyloidiasis/index.html

Figure 1.2 The life cycle of *S. stercoralis*

1.3.3.1 Autoinfection

Autoinfection is defined as the ability to replicate and multiply within the same host in the absence of external sources of infection (Gillespie and Pearson, 2001). In the *Strongyloides* genus, *S. felis* was found to have this trait as well as *S. stercoralis* (Speare and Durrheim, 2004). As mentioned previously, autoinfection may arise in one of two ways: i) the filariform larvae penetrate and lodge in the intestinal mucosa (internal autoinfection), or ii) the filariform larvae re-invade the bowel or perianal skin (external autoinfection) (Liu, 2012; Brooker and Bundy, 2009). The capability to establish a new cycle of repeated endogenous infection in the human host means the infection can persist for up to 75 years in immunocompetent individuals (Prendki *et al*., 2011). In immunocompromised individuals, it can lead to possible multiplication causing a tremendous increase in the worm burden, which in turn, leads to hyperinfection or disseminated syndrome (Liu, 2012).

1.4 Epidemiology

Strongyloidiasis is endemic worldwide; it can be found in countries with hot and humid climates but predominantly in resource-poor countries with inadequate sanitary conditions. The current prevalence of strongyloidiasis is estimated to be around 100-300 million cases (Taylor *et al*., 2014). This prevalence varies substantially between countries and continents due to ecological, socio-cultural, and economic factors, in addition to the type of diagnostic method and the number of studies undertaken. A review study conducted by Schär *et al*. (2013) reported a countrywide prevalence of strongyloidiasis based on three different subsets: community-based studies, hospital-based studies, and studies on refugees and immigrants.

Community-based studies have revealed that Brazil and Thailand are endemic countries for strongyloidiasis, and these two countries possess reliable and consistent data on the infection, with the proportion of the infection reported as being 13% and 23.7% respectively (Schär *et al*., 2013). One of the biggest community-based studies was conducted in Africa, and involved screening 20,250 individuals across 216 villages; the study revealed an infection rate of 11.6% among the community (Schär *et al*., 2013; Yelifari *et al*., 2005). Meanwhile in Japan, strongyloidiasis was found in older persons, with sustained infection, probably due to auto-infection, contributing to an overall infection rate of 16.4% in an endemic area of Okinawa prefecture (Arakaki *et al*., 1992). In a study conducted by Steinmann *et al*. (2007), the reported prevalence of strongyloidiasis in China was 11.7%, in which the endemic areas were found to be mostly farming communities. Based on the results of five prevalence studies, the reported prevalence of strongyloidiasis in Australia was found to be in the range of 0% to 60%, with screening taking place among the aboriginal communities in northern Australia (Johnston *et al*., 2005).

A hospital-based study analysing 37,621 laboratory specimens over a period of two years in the Campinas City region in Brazil demonstrated an overall prevalence rate of 10.8% (Rossi *et al*., 1992), while a prevalence rate of 11.2% in India was obtained from nine hospital-based prevalence studies, five of which focused on the screening of HIV/AIDS patients (Schär *et al*., 2013).

In the US, 347 deaths related to strongyloidiasis were reported from 1991 to 2006; this figure is equivalent to 14-29 deaths per year (Croker *et al*., 2010). The prevalence studies in Americas were well covered; two thirds of the epidemiologic studies in America focused mainly on refugees and immigrants. Similarly, seroprevalence studies in European countries focused on refugees, immigrants, and

travellers . In the United Kingdom, it is believed that *S. stercoralis* infection might have persisted over a long period, since many of the infections were identified among World War II veterans who had not left the country in the 60 years following their return from deployment in Southeast Asia (Schär *et al*., 2013). The reported prevalence in the UK was 12% (Gill *et al*., 2004). In Canada, a strongyloidiasis prevalence rate of 11.8% was reported among Vietnamese refugees, and 76.6% among Cambodian refugees (Gyorkos *et al*., 1990). Screening of 5,518 female housekeepers from different Asian countries working in Saudi Arabia revealed an overall prevalence of 0.6%, of which 0.4% were Filipinos, 0.5% Indonesians, 1.5% Sri Lankans, 2.6% Indians and 3.4% Thais (Madani and Mahfouz, 1995).

South-East Asia (SEA) possesses climatic, ecological, and socio-economic conditions that favour the transmission of *S. stercoralis.* Yet, information on the occurrence of strongyloidiasis from these countries is relatively scarce. In a systematic literature review of all peer-reviewed research articles published over the last 25 years conducted by Schär *et al.* (2015), they reported that the prevalence of *S. stercoralis* in Thailand ranged from 0.1% to as high as 38.8% when the infection was screened for among the general population, i.e. villagers and school-children. Thailand had by far the most extensive studies on the prevalence of strongyloidiasis among any other SEA countries. Meanwhile, a cross-sectional study carried out by Khieu *et al.* (2014) in rural Cambodia revealed that strongyloidiasis is highly prevalent among the general Cambodian population, with a prevalence rate of 44.7%. It is likely that the examination of multiple stool samples, the range of diagnostic methods employed, and poor sanitary conditions are the main factors that contributed to the high prevalence rate reported in this area. Similar reasons were also likely to be the main cause of the high prevalence rate of strongyloidiasis (41%) reported in

Lao PDR (Vonghachack *et al*., 2015). Seven screening studies conducted in Indonesia, covering the areas of Flores Island, Bali, and Irian Jaya Island, reported a prevalence rate lower than 1.0% to 1.6% (Schär *et al*., 2015). This low reported prevalence is believed to be due to the use of not highly sensitive diagnostic method (i.e. Harada-Mori technique) and the limited presence of *S. stercoralis* larvae. Meanwhile, no *S. stercoralis* infection was found in a study conducted in Vietnam. However, since the prevalence of hookworm infection was reported to be 28.6%, it is believed that strongyloidiasis is probably also present in Vietnam (Hung *et al*., 2005). No published report could be found on the prevalence rate of strongyloidiasis in Singapore, Brunei, Myanmar, and the Philippines (Schär *et al*., 2015).

In Malaysia, the first review case of a patient with gastric strongyloidiasis was histologically documented by Shekhar *et al*. (1997). Kuze *et al*. (2010) published a paper on the discovery of the rhabditoid larvae of *Strongyloides* sp. and *S. fuellerborni* from the faeces of orang utans in Sabah. Studies on the prevalence of *S. stercoralis* infection in this country is very limited; only four studies were conducted from 1997 to 2013, two of which studied the minority community of Orang Asli in rural areas and the rest were hospital-based studies in Sarawak and Kelantan. Rahmah *et al*. (1997) reported a low prevalence (1.2%) of strongyloidiasis among the Orang Asli minority community in Kelantan. In the same minority community, Ahmad *et al*. (2013) found no *S. stercoralis* larvae in 54 coprologically analyzed stool samples; however, serological examination of the corresponding serum samples revealed a prevalence of 31.5%. In a hospital-based study among patients with gastrointestinal symptoms in an endemic area of STH Sarawak, Basuni *et al*. (2011) reported a prevalence of strongyloidiasis to be 39.0% using pentaplex-PCR. Investigation of *S. stercoralis* infection among cancer patients in a major hospital in

Kelantan using several detection methods by Zeuter *et al.* (2014) revealed a seroprevalence of 3.1% when measured by IgG4-ELISA, 0.5% by microscopy and 1.6% by real-time PCR.

Figure 1.3 shows the prevalence of strongyloidiasis worldwide. It is noted that the high prevalence rates of *Strongyloides* infection were noted in areas where rigorous diagnostic approaches were engaged in the studies, be it due to multiple diagnostic methods or the large numbers of samples examined. In contrast, the low prevalence reported in certain areas might be due solely to the low sensitivity of the diagnostic method employed. This suggests that the prevalence of strongyloidiasis worldwide might be underestimated, as research into prevalence is highly dependent on the factors mentioned above. Therefore, a more sensitive and specific diagnostic method is required for an accurate measurement of prevalence as well as mapping the endemicity of the infection.

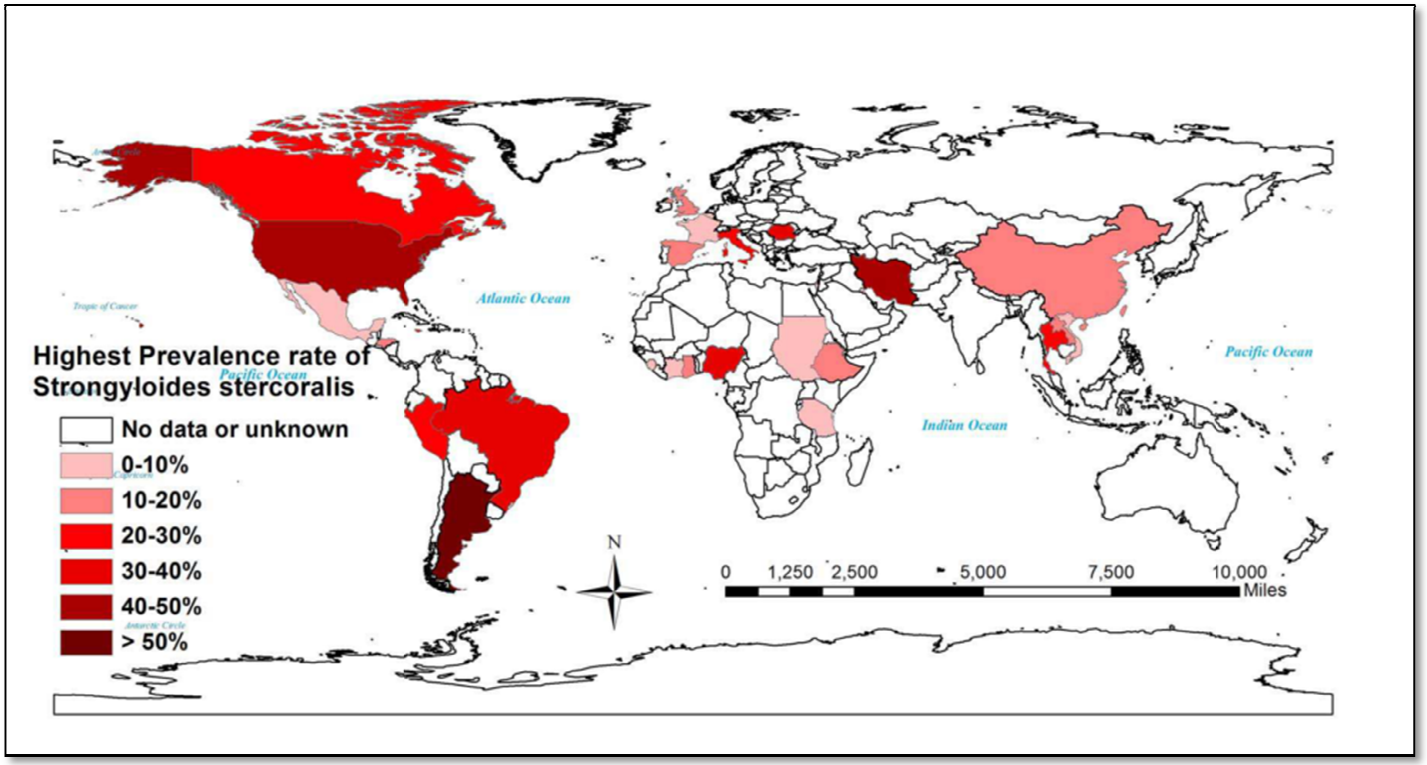


Figure 1.3 Worldwide prevalence of strongyloidiasis Puthiyakunnon *et al*., 2014

1.5 Transmission of the infection

1.5.1 Horizontal transmission

Humans are generally infected by *S. stercoralis* transcutaneously when the infectious filariform larvae in faecally contaminated soil enter the skin or mucous membranes during agricultural, recreational, or domestic activities (Ramanathan and Nutman, 2008). There are also cases of infection induced by the oral administration of water contaminated with filariform larvae (Keiser and Nutman, 2004) or the ingestion of contaminated food (Wirk and Wingard, 2009). Human-to-human transmission is uncommon; however, such transmission has been described among homosexual men (Simon, 2009). A group of cases were also reported in institutionalized individuals with mental retardation, suggesting that nosocomial transmission could possibly occur (Keiser and Nutman, 2004).

1.5.1.1 Soil transmission

Humans are prone to *S. stercoralis* infection when the person is exposed to contaminated soil harbouring infective filariform larvae. Thus, visiting an endemic area becomes the largest risk factor for acquiring *S. stercoralis* infection. Two cases of acute strongyloidiasis infection were reported in Italian tourists after visiting beaches in Koh Samui Island, Thailand (Angheben *et al*., 2011). Meanwhile, bathing in the rivers and consuming non-drinking water has been shown to have a significant association with strongyloidiasis (Herrera *et al*., 2005). Farmers and coal miners are the two occupations with the highest risk of getting the infection, while children are prone to this disease due to their behaviours, which favour the transmission of the soil-based helminths – dirty hands, mouth contact, and going bare foot when playing on contaminated ground (Liu, 2012).

1.5.1.2 Oral route of transmission

The *Strongyloides* larvae have been found contaminating local vegetation, by which the infection can be transmitted orally to humans. Some people especially Kelantanese have the custom of eating raw vegetables and herbs, i.e. *daun kesum* (Vietnamese mint), *pegaga* (*Centella asiatica*), and water spinach (*Ipomoea aquatic*); these plants are collectively known as *ulam* (Zeehaida *et al*., 2011). Most of these *ulam* grow in areas near the drains, a place that favours the growth of the parasite. Zeehaida *et al.* (2011) revealed the presence of live rhabditiform *S. stercoralis* larvae in the water samples used to wash these vegetables, highlighting the possibility of oral route transmission to the public, vegetables sellers and food handlers.

1.5.1.3 Person to person transmission

The possibility of the transmission of larvae from one person to another via direct contact is low; hence, few reports are available. In 1982, Grove conducted a survey investigating the possible transmission of *S. stercoralis* from 24 men who had been infected with strongyloidiasis for many years to their spouse. The results showed that none of the wives were infected, suggesting that the risk of transmission is small. Indeed, no evidence of transmission was found in medical staff in charge of patients with disseminated strongyloidiasis (Sugiyama *et al*., 2006; Maraha *et al*., 2001). However, the risk of infection could still be present if safety precautions are not strictly followed. For instance, Czachor and Jonas (2000) reported a case of *S. stercoralis* infection in an 84-year old wife who had routinely provided personal care and attended to the secretions of her ill husband, who had hyperinfestation syndrome.

The wife presented with uncharacterized symptoms two weeks after her husband's death.

The likelihood of transmission among homosexuals is greater (Grove, 1982). A case was reported by Sorvillo *et al.* (1983) who identified the presence of *S. stercoralis* in the faeces of a homosexual patient and his contacts. Thus the risk of person-to-person transmission among homosexuals should not be ignored.

1.5.2 Vertical transmission

The occurrence of vertical transmission of strongyloidiasis in humans is unknown. In a study conducted by Shoop *et al.* (2009), they examined the possibility of the vertical transmission of *S. stercoralis* in three female dogs at a different stages of the reproductive cycle (preconception, gestation, and postpartum) which were injected with *S. stercoralis* filariform larvae. None of the pups born to females infected before conception and during gestation harboured the larvae, thus showing that transmission through prenatal pathways does not occur. However, live filariform larvae of *S. stercoralis* were found in milk samples taken from lactating female dogs, suggesting that transmission of the parasite through transmammary route. The larvae are able to become dormant in the tissue, and this was found to be the key for transmammary transmission. The infective L3 presumably arrest their development and migration in the mammary glands, and then reactivate at lactation (Liu, 2012).

Recently, a single case of a pregnant woman who died due to strongyloidiasis has been reported by Buresch *et al*. (2015). The woman, who was at week 25 of gestation, was given a corticosteroid to resolve the acute abdominal pain and abnormal foetal heart tracing. Unfortunately, she developed septic shock, due to hyperinfection causing stillbirth to occur, and finally she succumbed to the disease.

1.6 Clinical manifestation of strongyloidiasis

1.6.1 Acute

The primary infection with *S. stercoralis* is initiated by penetration of the infective filariform larvae through the intact skin. This early stage of infection is usually asymptomatic, or it may manifest as mild larva currens, an allergic reaction due to migration of the filariform larvae (Mahmoud, 1996). The migration can progress as quickly as 10 cm/hr (Satoskar *et al*., 2009). Slight haemorrhage, swelling, and itching are sometimes noted at the site of larva entry; these symptoms can occur almost immediately and can last for several weeks (Keiser and Nutman, 2004; Freedman, 1991).

After penetrating the skin, the infective filariform larvae enter the blood, and they are then transported to the lungs via the pulmonary circulation. At this time, patients usually suffer from symptoms that resemble Loffler's syndrome, such as coughing, shortness of breath, wheezing, and transient pulmonary filtrates (Mahmoud, 1996). Inside the lungs, the larvae penetrate the alveoli and migrate upwards to the tracheobronchial tree; they are then swallowed entering the gut, where they complete their maturation. These larvae will then moult to become adult female worms and lodge in the submucosal tissues inside the proximal small intestine (Simon, 2009). At this stage, abdominal symptoms and signs such as indigestion, cramping abdominal pain, and diarrhoea with malabsorption and weight loss, have been commonly observed (Mahmoud, 1996) about two weeks after infection, with the larvae detectable in the stool after three to four weeks (Keiser and Nutman, 2004). All of these may be misdiagnosed as being due to irritable bowel syndrome (Grove, 1995).

1.6.2 Chronic

Unresolved acute infection can lead to the development of chronic infection, causing a variety of manifestations, most commonly infections to the skin and gastrointestinal system (Grove, 1995). Meanwhile, chronic infection, without prompt diagnosis and proper treatment can lead to severe complicated strongyloidiasis characterized by a wide range of complicated forms of disease, including gastrointestinal, pulmonary, and neurological complications and other presentations such as urinary tract infections and pelvic inflammation (Grove, 1995). The major target areas are the bowel, lungs, and central nervous system. Usually, this leads to presence of secondary bacterial infection, which increases the risk of mortality in patients not only due to the hyperinfection syndrome, but also due to the underlying condition which predisposes to dissemination (Grove, 1995).

Autoinfection is the main reason for the persistence of this organism in the host for decades. A more severe form of autoinfection is the hyperinfection syndrome (HS). The hallmark of HS is an increase in the number of larvae found in the stool and/or sputum along with a clinical manifestation that is confined to the usual migratory pathway of the parasite, i.e. the respiratory and gastrointestinal systems and the peritoneum (Liu, 2012). Patients with HS at the gastrointestinal sites usually suffer from clinical manifestations, such as cramping abdominal pain, watery diarrhoea, nausea, vomiting, gastrointestinal bleeding, and weight loss. Meanwhile, hyperinfection in the extraintestinal sites include coughing, wheezing, and pulmonary haemorrhaging with diffuse bilateral infiltrates seen on the chest x-rays (Liu, 2012).

The key factors contributing to HS are drug-induced or concurrent immunosuppressive conditions, such as solid tumours, corticosteroid use, HTLV-1

and HIV infection, or Hodgkin's lymphoma (Gillespie and Pearson, 2001). HS occurs in 1.5-2.5% of patients with strongyloidiasis, contributing to a 15% mortality rate among HS patients, which increases to 87% when there is dissemination (Marcos *et al*., 2008; Vadlamudi *et al.*, 2006).

The uncontrolled HS may result in the dissemination and massive migration of the infective larvae outside the usual migration pattern to the other extraintestinal organs; this usually leads to a fatal outcome. The larvae are found in other organs, including the liver, the kidneys, and the central nervous system (Simon, 2009). During the migration, the larvae facilitate the translocation of intestinal bacteria, such as *Streptococcus bovis, Escherichia coli, Streptococcus fecalis, Klebsiella pneumonia*, or *Enterobacter sp.* to other locations of the body (Liu, 2012). These gut flora invade the host tissues either through the penetration of infective larvae from the bowel lumen or through the damaged intestinal epithelium causing concomitant secondary disease, such as septicaemia, meningitis, liver abscess, and pancreatitis (Mahmoud, 1996). Cases of disseminated strongyloidiasis have been reported to be associated with systemic erythematosus (SLE) (Setoyama *et al*., 1997), nephrotic syndrome (Morimoto *et al*., 2002), and malignant tumours (Genta *et al.*, 1989).

1.7 Strongyloidiasis in patients with associated problems

1.7.1 Immunosuppressed patients

Immunosuppressed patients are among the populations most at risk of developing the life-threatening clinical syndromes associated with strongyloidiasis, i.e. HS or dissemination (Marcos *et al*., 2011). These patients include those with human T-cell lymphotrophic virus type I (HTLV-I) infection, patients with hematologic malignancies, and patients who have received systemic corticosteroids or an

allogeneic hematopoietic stem cell transplantation (HSCT) (Marcos *et al*., 2008). Indeed, leukaemia and lymphoma account for up to 90% of the cases of malignancy-associated severe strongyloidiasis (Schaffel *et al*., 2001; Igra-Siegman *et al*., 1981).

In the US, the overall frequency of infection amongst 322,593 cancer patients diagnosed between 1971 and 2003 was found to be 0.8 per 10,000 patients and to be 2.0 per 10,000 among patients with leukaemia (Safdar *et al*., 2004). Meanwhile, in Malaysia, *S. stercoralis* infection was detected in 4.2% of cancer patients, thus highlighting the high association of strongyloidiasis among immunosuppressed cancer patients (Zueter *et al.*, 2014).

In general, immunosuppression develops one to three weeks after the administration of the immunosuppressant (Thackery, 2002), and signs and symptoms of strongyloidiasis are seen in immunosuppressed patients as early as 20 days and as late as several years after the onset of steroid therapy (Keiser and Nutman, 2004). In a high-dose corticosteroid treatment, it can take less than ten days to transform a previously clinically silent undetected infection into overwhelming dissemination (Genta, 1992). Patients of these cases mostly received glucocorticoids (one of the corticosteroids), which is the most widely used treatment and the most specifically associated with transforming chronic strongyloidiasis to hyperinfection (Keiser and Nutman, 2004).

The link between corticosteroid therapy and strongyloidiasis has been widely reviewed. According to Corrigan (1999), the corticosteroids have the effect of reducing the levels of circulating eosinophils by inhibiting their proliferation and increasing apoptosis, in addition to inducing cell death in immature lymphocytes (Marcos *et al.*, 2008). It was also been hypothesized that corticosteroids may directly affect the female worms, accelerating the transformation of rhabditiform to invasive

filariform larvae (Genta, 1992), or rejuvenating reproductively latent adult females (Keiser and Nutman, 2004; Mansfield *et al*., 1996).

The use of other immunosuppressants other than corticosteroids has also been associated with a number of cases of hyperinfection, i.e. vinca alkaloids (Jamil and Hilton, 1992), cyclosporine (Palau and Pankey, 1997), azathioprine (Weller *et al.*, 1981), and VP16 (Tabacof *et al*., 1991).

1.7.2 Association with solid organ transplantation

More than one-half of cancer patients who had strongyloidiasis also had an underlying solid-organ malignancy (Safdar *et al*., 2004). Post-transplantation tropical infection is a major risk in the organ recipient, which is frequently misdiagnosed by clinicians. It is thought that most *Strongyloides* infections in organ transplant recipients are caused by the reactivation of chronic infection after the initiation of immunosuppressive therapy or donor-derived infection.

The incidence of *Strongyloides* infection post-transplantation in organ recipients is increasing annually due to the increase in international travel and the rising number of transplant procedures taking place in tropical countries. The most frequent occurrence of *Strongyloides* infection was found in renal transplantation (Marcos *et al*., 2008), with one of the earliest cases described in 1971 (Snydman *et al*., 2009; Fagundes *et al.*, 1971). Other cases of strongyloidiasis have also been reported in recipients of intestinal transplant (Patel *et al*., 2008), liver transplant (Rodriguez *et al*., 2009), heart transplants (Ziad El Masry and O'Donnell, 2005) and lung transplants (Balagopal *et al*., 2009). Two cases of symptomatic chronic strongyloidiasis in immunosuppressed children following treatment for solid organ malignancies were reported in Malaysia by Norsarwany *et al*. (2012).