OPTIMIZING THE PRODUCTION OF SHORT-PEPTIDE TAGGED SS3A RECOMBINANT PROTEIN AS A POTENTIAL SEROLOGICAL BIOMARKER FOR STRONGYLOIDIASIS

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by

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"In the name of God, the Most Gracious, the Most Merciful"

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Ml	Microliter
µm	Micron
µg	Microgram
APS	Ammonium persulfate
bp	Base pair
BSA	Bovine serum albumin
CDC	Centre for Disease Control and Prevention
Cdna	Complementary deoxyribonucleic acid
CV	Cut off value
ECL	Chemiluminescence
e.g.	For example
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FPLC	Fast protein liquid chromatography
g	Gram
GST	Glutathione S-transferase
His	Histidine
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HRP	Horesradish peroxidase
HTLV-1	Human T-cell lymphotropic virus
i.e.	id est (that is)
IEX	Ion exchange chromatography
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgG	Immunoglobulin G4
INFORMM	Institute for Research in Molecular Medicine
IPTG	Isopropanyl-beta-D-thiogalactopyranoside
kDa	Dalton
LAMP	Loop-mediated isothermal amplification
L	Liter
LB	Luria-Bertani
LIPS	Luciferase immunoprecipitation system
M	Molar
MALDI TOF/TOF	Matrix-assisted laser desorption/ionization time of flight/time of flight
mg	Milligram
Ml	Milliliter
Mm	Millimolar
MW	Molecular weight
MWCO	Molecular weight cut-off
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometer

OD	Optical density
OFFGEL	a fractionator to separate proteins based on P*i* value
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Ph	Potential hydrogen
P*i*	Isoelectric point
Qpcr	Real-time PCR
RCF (x *g*)	Relative centrifugal field
RC DCTM	Reducing agent detergent compatible
SEC	Size exclusion chromatography
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB	Terrific broth
TBS	Tris-buffered saline
TBS	T tris-buffered saline with tween-20
TEMED	Tetramethyl ethylenediamine
USM	Universiti Sains Malaysia
UV	Ultraviolet
V	Voltage/volts
v/v	Volume/volume
WHO	World health organization

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PENGOPTIMUMAN PENGELUARAN REKOMBINAN SS3A BESERTA PEPTIDA PENDEK SEBAGAI PENANDA BIO SEROLOGI UNTUK STRONGYLOIDIASIS

ABSTRAK

Strongyloides stercoralis ialah nematode yang patogenik bagi manusia dengan keupayaan unik untuk autoinfeksi menyebabkan penyakit parasit yang dipanggil strongyloidiasis. Walaupun World Health Organisation (WHO) menyenaraikan jangkitan ini sebagai salah satu jangkitan tropika terabai, jangkitan ini mempunyai taburan kes di seluruh dunia dengan kira-kira 613.9 juta kes, kebanyakannya di negara- negara bercuaca tropika. Jangkitan terhadap manusia berlaku apabila larva filariform *S. stercoralis* dari tanah yang tercemar menembusi lapisan kulit melalui sentuhan langsung, bergerak ke mulut melalui aliran darah sebelum ditelan dan tinggal di dalam usus perumah. Di dalam usus larva dewasa betina akan menghasilkan telur secara partenogenetik, meneruskan kitaran hidup tanpa meninggalkan badan perumah melalui proses yang dipanggil autoinfeksi. Secara umumnya, gejala selepas jangkitan berbeza dalam dua cara. Bagi individu imunokompeten, perumah selalunya menunjukkan gejala yang minima atau tanpa gejala (asimtomatik) dan ini menyebabkan jangkitan sepanjang hayat manakala pada individu yang mempunyai imuniti terjejas, jangkitan yang tidak dikawal cenderung untuk menyebabkan sindrom hiperjangkitan, keadaan di mana larva membiak secara berlebihan dan tersebar ke organ-organ termasuk paru-paru, hati dan otak. Akibatnya, kematian bagi kumpulan individu ini boleh menyebabkan kadar kematian meningkat kepada 85% ke 100%. Walaupun ancaman adalah tinggi, diagnosis strongyloidiasis adalah sukar kerana jumlah beban nematod yang rendah dan outputnya yang bersela. Walaupun pemeriksaan secara mikroskopik kekal sebagai

kaedah piawai yang kontroversi, diagnosis yang lebih baik dicapai melalui ujian pengesanan dengan pendekatan diagnostic serologi dan/atau molekul. Dalam kaedah terkini serodiagnosis strongyloidiasis, rekombinan protein telah diguna pakai menggantikan penggunaan antigen asli parasit, biarpun ketersediaan protein yang berpotensi untuk kegunaan diagnostik masih terhad. Dalam kajian ini, kami mencadangkan varian antigen rekombinan Ss3a yang lebih baik dalam vektor pET32(a) untuk mengesan penyakit strongyloidiasis. Kajian ini menfokuskan kepada mengoptimumkan dua varian protein rekombinan Ss3a iaitu rSs3a7D dan rSs3a7K, masing-masing mempunyai tujuh residu asid aspartik dan tujuh residu lisin yang dilekatkan pada N-terminal. Dalam proses ekpresi protein, parameter-parameter yang optimum adalah seperti yang berikut: 0.5 mM kepekatan IPTG, empat jam tempoh inkubasi selepas induksi, dan sel hos BL21(DE3) bagi rSs3a7D dan 0.5 mM kepekatan IPTG, empat jam tempoh inkubasi selepas induksi, dan sel hos C41(DE3) bagi rSs3a7K. Selain itu, dalam penulenan kaedah kromatografi affiniti, kepekatan penimbal pembasuh sebanyak 20 mM, 30 mM dan 40 mM telah dipilih sebagai strategi pencucian protein yang optimum dan HisPur^{TM} Ni-NTA Superflow resin (Qiagen GmbH, Hilden, Germany) sebagai resin yang optimum bagi penulenan protein. Kemudian, kedua-dua protein ini dimajukan bagi penilaian antigenik menggunakan 40 serum individu dengan IgG4 -HRP tikus anti-manusia sebagai prob dalam analisis westen blot. Keputusan menunjukkan bahawa rSs3a7D dan rSs3a7K masing-masing mempunyai spesifisiti 60% (n=20) dan 85% (n=20), manakala sensitiviti bagi kedua-dua adalah 80% (n=20). Berdasarkan ini, rSs3a7K dipilih sebagai penanda biologi yang berpotensi dalam mengesan strongyloidiasis. Langkah penulenan selanjutnya diteruskan untuk meningkatkan ketulenan rSs3a7K dengan menggabungkan kromatografi pertukaran ion dan pegecualian saiz, namun

penambahbaikan ini adalah tidak memberangsangkan di mana hasil rSs3a7K didapati merosot dan memberikan kepekatan akhir yang rendah. Identiti protein rekombinan Ss3a7K turut disahkan melalui analisa MALDI TOF/TOF.

OPTIMIZING THE PRODUCTION OF SHORT-PEPTIDE TAGGED SS3A RECOMBINANT PROTEIN AS A POTENTIAL SEROLOGICAL BIOMARKER FOR STRONGYLOIDIASIS

ABSTRACT

Strongyloides stercoralis is a human-pathogenic nematode with a unique ability to autoinfect causing a parasitic disease called strongyloidiasis. Although listed as one of the neglected tropical diseases by World Health Organisation (WHO), the infection has a worldwide distribution with approximately 613.9 million cases mostly in tropical countries. Human infection occurs when the infective filariform *S. stercoralis* larvae in contaminated soil penetrate the intact skin through direct contact, travel to the mouth through bloodstream before it gets swallowed and resides in the gut. In the gut, female adult larvae produce eggs parthenogenetically, continuing their life cycle without having to leave the host's body through a process called autoinfection. In general, the post-infection symptoms vary in two different ways. In immunocompetent individuals, hosts usually exhibit minimal to no symptoms (asymptomatic) and causes a life-long infection whereas in immunosuppressed individuals, unchecked infection is highly inclinative towards developing hyperinfection syndrome, an event where the larvae over-proliferate and disseminate to organs including the lung, liver, and brain. Consequently, the fatality in the latter group with disseminated infection can cause the mortality rate to rise to 85%-100%. Despite the threat it imposed, diagnosing strongyloidiasis is difficult in accord to the nematode's low burden and intermittent output. While microscopic examination remains a "controversial" gold standard method, improved diagnosis is achieved through confirmatory assays with serological and/or molecular diagnostic

approaches. In the current serodiagnosis of strongyloidiasis, recombinant proteins have been adopted in place of the use of native parasite antigens, although the availability of diagnostically potential proteins are still limited. In this study, we propose an enhanced variant of recombinant protein Ss3a in pET32(a) vector to detect strongyloidiasis. This study focused on optimizing two variants of Ss3a recombinant protein namely rSs3a7D and rSs3a7K, with seven residues of aspartic acid and lysine respectively attached at the N-terminal of the recombinant. During protein expression, the optimized parameters are as follow: 0.5 mM IPTG concentration, four hours post induction incubation period and showed higher protein yield in BL21(DE3) for rSs3a7D whereas 0.5 mM IPTG concentration, four hours post induction incubation period and showed higher protein yield in C41(DE3) for rSs3a7K. On the other hand, in purification using affinity chromatography, a washing pattern of 20 mM, 30 mM, 40 mM of washing buffer was chosen to be the most optimal washing strategy for both variants, and HisPur^{TM} Ni-NTA Superflow resin (Qiagen GmbH, Hilden, Germany) was chosen as the optimal resin for protein purification. Thereafter, the two variants were subjected to antigenicity evaluation using 40 individual serum samples with mouse anti-human IgG4-HRP as probe in western blot analysis. Results showed that rSs3a7D and rSs3a7K had a specificity of 60% (n=20) and 85% (n=20), respectively, while both sensitivities were 80% (n=20). Based on this, rSs3a7K was chosen as the potential biomarker in detecting strongyloidiasis. Further purification steps were attempted to improve the purity for rSs3a7K by incorporating ion exchange chromatography and size exclusion chromatography, but the improvement was not encouraging, where the rSs3a7K yield was found to deteriorate and gave to a low final concentration. The identity of recombinant Ss3a7K was also confirmed by MALDI TOF/TOF analysis.

CHAPTER 1

INTRODUCTION

1.1 Human strongyloidiasis: An overview

Strongyloides stercoralis is a human parasitic nematode effectuating a deadly, yet mysterious infection called strongyloidiasis. The common symptoms of strongyloidiasis are categorized into dermal, gastrointestinal (GI) and pulmonary. GI symptoms such as diarrhoea, anorexia, and abdominal discomfort usually appear two weeks after infection, with larvae appearing in stool three to four weeks later. The onset of pulmonary symptoms such as tracheal discomfort, cough, and bronchitis is significantly earlier than the onset of GI symptoms as larvae travels up to the lung first after skin penetration (Puthiyakunnon *et al.*, 2014). Unlike other nematodes, this parasite is able to undergo a process called autoinfection, which allows infective larvae to penetrate the host and reside within while perpetuating another infective cycle (D. Buonfrate *et al.*, 2015). In this state, the larvae penetrate the intestinal wall or perianal region and continue to multiply at low levels of infection within the same host (Mahmoud, 1996). For this reason, infection in immunocompetent individuals tend to be on a life-long scale and persistently remain undetected for many decades after its initial exposure (Varatharajalu & Rao, 2016), up to 75 years in one case report (Ravanini *et al*., 2011). However, in individuals with compromised immune system, autoinfection can be worsened causing the infection to undergo hyperinfection, due to an extensive increased load of the parasite that are free to disseminate into other body organs, a medical state that is almost always fatal (Mirdha, 2009; Varatharajalu & Rao, 2016; Terefe *et al*., 2019).

This infection was initially reported to have an estimated prevalence of 30 - 100 million people worldwide (Khadka *et al*., 2018; Olsen *et al*., 2009; Puthiyakunnon

et al., 2014), however the estimation was severely undervalued and rather obsolete considering the data was collected between the year 1989 to 1996 (Mun *et al*., 2013). This figure was cited by many articles and was generally based on surveys using common procedures of microscopic examination such as Kato-Katz technique to diagnose the infection (de Paula *et al*., 2015; Greaves *et al*., 2013; Montes *et al*., 2011; Norhayati *et al*., 2003; Puthiyakunnon *et al*., 2014; Shafik *et al*., 2018; Siddiqui & Berk, 2001; Varatharajalu & Kakuturu, 2016). Nonetheless, these methods rely heavily on the presence of larvae on stool samples, for which is poorly sensitive and often yields false negatives (Viney & Lok, 2015). In recent years, alternative diagnostic techniques have surfaced and offer compelling advantages as opposed to the existing conventional techniques, despite many modifications have been done to the procedures (D. Buonfrate *et al*., 2015). Despite these advances, the current prevalence worldwide is proposed to be on the scale of 613.9 million reported cases (Buonfrate *et al*., 2020) from just 370 million in less than 10 years ago (Aupalee *et al*., 2020; D. Buonfrate *et al*., 2015; Mun *et al*., 2013).

The forging of serology-based and molecular-based techniques have tremendously uplifted the perspective of diagnosing strongyloidiasis. On 29 th September 2020, the World Health Organization (WHO) held a virtual meeting to select the optimal diagnostic approach or combination strategies for *S. stercoralis* infection control and their panels' recent discussion concluded that the optimal initial approach for strongyloidiasis is by serology, followed by additional assays in subsequent phases of infection (World Health Organization, 2020). However, no single serological test was shown to be particularly accurate. The advances also hit a drawback when problems related to cross-reactivity seem to surface, especially in areas co-endemic with other helminthic infections (D. Buonfrate *et al*., 2015; Olsen *et al*., 2009). Above all, the default where a go-to guideline should be anchored is lacking

(D. Buonfrate *et al*., 2015). While these techniques complement and bridge the gaps left unfilled by the existing diagnostic methods, such quantum leap can be very costly and not user friendly. This study aims to provide an insight into current diagnostic methods and propose a new recombinant protein as a potential biomarker to diagnose *S. stercoralis.*

1.2 The organism: *Strongyloides stercoralis*

1.2.1 Taxonomy

Strongyloides is a genus classified under the order of Rhabditida and family of Strongyloididiae (Grove, 1996) where it comprises more than 50 species known to be an obligate gastrointestinal parasite in vertebrates (Viney & Lok, 2015). In studied cases, most species evidently are able to infect one or no more than just a few host species. Even so, vast majority of these species do not infect humans. Leading from here, there are two species of soil-transmitted helminths from the genus *Strongyloides* that are known to parasitize humans namely S. *fuelleborni* and the pathogenic species *S. stercoralis* (Olsen *et al.*, 2009). While *S. fuelleborni* is usually common in non-human primates, human infections have also been reported in multiple sporadic cases from Central Africa, Papua New Guinea, and Southeast Asia (Olsen *et al*., 2009; Varatharajalu & Rao, 2016). However, *S. stercoralis* on the other hand has accounted for more than 90% of the cases (Varatharajalu & Rao, 2016) and has a cosmopolitan dispersal pattern around the globe whilst being endemic to tropical and subtropical regions (Viney & Lok, 2015).

1.2.2 Life cycle

The life cycle of *S. stercoralis* is unique and rather complicated with the alternation between free-living and parasitic cycle (Figure 1). As mentioned, there are two parts of pathways, those being the free-living cycle (the non-infective cycle) and the host-mediated parasitc cycle (infective cycle) (Grove, 1996; Keiser & Nutman, 2004; Olsen *et al*., 2009; Siddiqui & Berk, 2001). *S. stercoralis* is likely to be found in areas with poor sanitation and hygiene, where the soil is contaminated with fecal excretions (Greaves *et al*., 2013). Under favorable conditions, the rhabditiform larvae

in the soil transforms into infective filariform larvae, which is then acquired by humans who come in contact percutaneously and henceforth, penetration process begins (Kassalik, 2011). The migration of the larvae via submucosa route has been traditionally described where they enter the venous circulation and travel all the way up to the lungs before they are expectorated to the pharynx and subsequently swallowed (Mahmoud, 1996). This traditional migratory route is now discerned to be as equally significant to another migration route which describes a direct penetration from the skin to the duodenum (Mahmoud, 1996).

Furtherance from there, the parthenogenetic females settle in the duodenal mucosa where they lay embryonated eggs which then hatch *in situ,* giving rise to a generation of rhabditiform larvae within the intestinal wall of the host (Siddiqui & Berk, 2001). The fresh larvae then travel into the lumen where they are either discharged together with feces or grow into filariform larvae which can re-infect the host's intestinal mucosa or the perianal skin to re-initiate another parasitic life cycle (Montes *et al*., 2011). By way of alternative, rhabditiform larvae which were excreted together with the feces will either mature into infectious filariform larvae or follow a free-living life cycle development in soil (Keiser & Nutman, 2004).

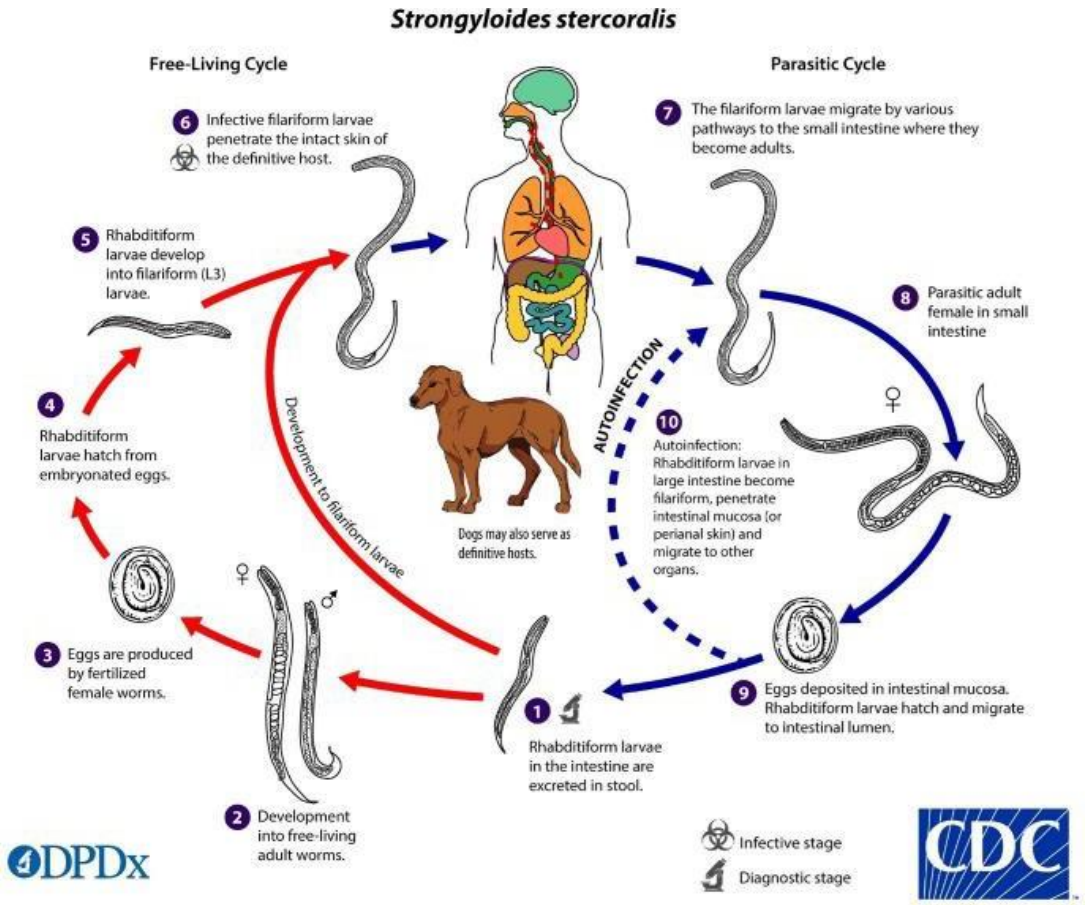

Figure 1.1 Life cycle of *Strongyloides stercoralis* incorporating both parasitic and free-living cycles. (Retrieved from Centers for Disease Control and Prevention website, 2019; https://www.cdc.gov/dpdx/strongyloidiasis/index.html)

1.2.3 Morphology

Both parasitic and free-living stages of *S. stercoralis* exhibit different morphological characters (Figure 2). The length of parasitic females are approximately 2 mm joints with blunt-ended tails, and an elongated, straight-sided esophagus, taking up almost one third of the body length (Viney & Lok, 2015). The worm is described as almost transparent, small and slender with a single cell-thick tube-like intestine that ends with a short rectum, and there is an opening at the anus near the tip of the tail (Grove, 1996). The body of *S. stercoralis* functions with two longitudinal excretory canals alongside the body length on each lateral side of the worm, this is then bridged with a transverse duct across the canals (Grove, 1996). Besides that, this worm has a didelphic ovary that opens at the vulva, this in which is positioned approximately two thirds of the whole-body length (Viney & Lok, 2015).

On the contrary, the free-living adult stages for both males and females are approximately 1 mm in body length where the female is slightly bigger than male (Viney & Lok, 2015). Free-living adults of *S. stercoralis* have thin cuticle which is transparent and has striation patterns whereas the esophagus is divided into three different segments: the anterior cylindrical procorpus, the lean isthmus and the rounded bulb on the posterior part of the body (Grove, 1996). The reproductive organ of the free-living female is almost similar to the parasitic female adult, except that it is slightly shorter (Little, 1966) and holds a higher density of eggs (Grove, 1996).

Contrastingly, the free-living male adult's body ends with a sharp pointed tail which bends on the anterior, hence giving the male worm a slightly 'J-shape' (Little, 1966). Alongside with this, male *S. stercoralis* has a straight tube as its reproductive organ, this in which has a blind-ended testis on the anterior end which merges into the vas deferens and then into the seminal vesicle (Grove, 1996). The mentioned organs

carry vital reproductive elements such as the spermatogonia, spermatocytes and sperms which open to the cloaca (Grove, 1996). Therein, the cloaca is attached to a pair of copulatory spicules which are used to lodge inside the female's reproductive organ during copulation (Grove, 1996).

Parasitic adult females are widely known to be the notorious face of this worm, therefore a number of unique features and characteristics have been extensively used for species identification (Viney & Lok, 2015). Some the features are the position of the ovaries, where they are wrapped over the intestine or in a parallel position to it and its forked tail at the bottom (Viney & Lok, 2015).

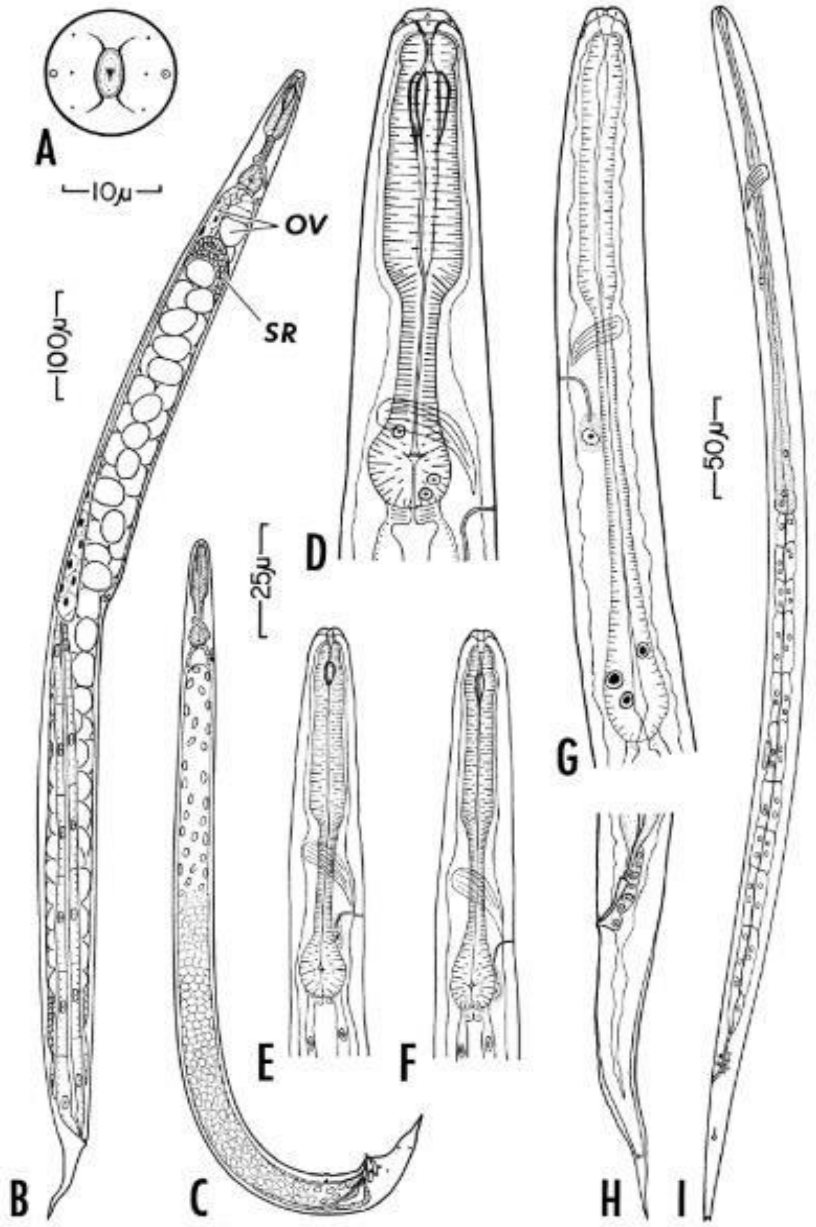

Figure 1.2 The morphological features of *S. stercoralis*. **A**. The apical view (*en face*) of the anterior side of free-living female adult. **B**. Free-living female worm on the lateral view (OV, ovary; SR, seminal receptacle which carries sperm). **C**. Free-living male worm. **D**. The anterior end of free-living female with detailed particulars of esophagus. **E**. First stage of larva in duodenal aspirate from humans. **F**. Rhabditiform maturing to the filariform stage, cuticle is seen to separate at the anterior end. **H**.Tail view of the second-stage larva, cuticle begins to separate. **I**. Filariform larva exhibiting the forked tail pattern. (From Little, 1966)

1.3 Clinical manifestation

Strongyloidiasis is a substantial public health threat with multiple magnitudes of severity and clinical syndromes (Khadka *et al*., 2018). Such presentations are categorized into four different stages namely: acute, chronic, hyperinfection and dissemination (Keiser & Nutman, 2004; Mejia & Nutman, 2013; Puthiyakunnon *et al*., 2014)

1.3.1 Acute strongyloidiasis

Acute strongyloidiasis is normally symptomatic and is closely linked to the migration of larvae towards the intestine (Mejia & Nutman, 2013). The first symptom exhibited can be as early as an immediate reaction as larvae penetrate the skin where this may persist up to a few weeks before the subsequent symptoms appear (Puthiyakunnon *et al*., 2014).

Pulmonary symptoms usually appear as coughs and irritations to the tracheal area due to the larvae travelling through the lungs whereas gastrointestinal symptoms encompass diarrhea, abdominal pain and constipation. These symptoms occur approximately two weeks after the initial infection, and larvae can be observed in fecal sample on the third to fourth week of manifestation (Keiser & Nutman, 2004).

However, symptoms of the acute stage could differ largely in duration, depending on a person's health status. For example, two cases of acute strongyloidiasis were reported with symptoms of fever, cough and eosinophilia which appeared at 11 to 14 days post-exposure, a time which was much shorter than expected as discussed in many scholarly articles (Caumes & Keystone, 2011). This differs from another case of acute stage infection involving a patient coming back from a vacation in Cuba and reported symptoms such as abdominal pain, rashes on skin and diarrhea four weeks after her return (Alabi *et al*., 2017). Another interesting

case reported involved two Italian tourists returning from Southeast Asia where only one of them showed presence of rhabditiform larvae in his stool samples after five stool samplings (Angheben *et al*., 2011). These cases are some of the examples that showed the diversity of manifestation and duration of the early symptoms, thus indicating the need for prompt case detection.

1.3.2 Chronic strongyloidiasis

At the onset of chronic stage of strongyloidiasis, the infection is often asymptomatic, and is usually accompanied by an increase in eosinophils. Rossi *et al*., (1993) reported that about 75% of infected individuals showed an increased level of total IgE surpassing 250 IU/ml (Caumes & Keystone, 2011; Mejia & Nutman, 2013). In immunocompetent individuals, the infection is either asymptomatic or shows minor clinical symptoms (Rossi *et al*., 1993) and this includes diarrhea, intermittent vomiting or constipation whereas dermatological symptoms entail a series of recurrent urticaria, larva currens, or purpuric rashes due to the migrating larvae throughout the body (Mejia & Nutman, 2013; Puthiyakunnon *et al*., 2014). The asymptomatic condition however can remain undetected in immunocompetent individuals until many decades with the current longest record stands at 75 years (Ravanini *et al*., 2011; Siddiqui & Berk, 2001). This is solely attributed to the ability of the parasite to perform autoinfective cycle as described before.

Adversely, different scenarios can be seen in immunosuppressed patients. Clinical presentations rely heavily on the interaction between the parasite and the immune response of the host, more specifically the Th2 cell-mediated immunity, thus defects on the cell mediated immunity are able to alter an asymptomatic clinical presentation into a fulminant and often lethal disease (Corti, 2016). Not to mention the ability of the parasite to perform autoinfection, hence given this opportunistic

condition where the host's body defense is suppressed, this would further augment the parasite load in the infected host (Ramanathan & Nutman, 2008), putting individuals with compromised immunity at higher risk of hyperinfection (Azizi *et al.*, 2017).

1.3.3 Hyperinfection syndrome

Hyperinfection syndrome usually affects patients with altered immune status where autoinfection is accelerated producing a colossal parasite load in the same migratory pattern in the host body (Puthiyakunnon *et al.*, 2014). This phenomenon is known as the hallmark of hyperinfection syndrome that results in severe gastrointestinal and pulmonary complications (Mejia & Nutman, 2013; Ramanathan & Nutman, 2008). There are a large number of published studies that described the unusual multiplication of infective larvae as a cause of hyperinfection and acts as a bridging point towards dissemination, and this is knowingly associated with immunocompromised group, although some researchers have also reported cases of hyperinfection in immunocompetent patients as well (Husni *et al.*, 1996).

Regardless, patients experiencing hyperinfection syndrome may still present eosinophilia in their blood count but more often than not, the count is usually suppressed and lessened, whereas in the case of patients showing an increase in peripheral eosinophilia in their blood count during hyperinfection are normally shown to have a better clinical prognosis (Keiser & Nutman, 2004). The important risk factors that contribute to developing this syndrome have been identified due to corticosteroid therapy, organ transplantation, alcoholism, and HIV infection (Puthiyakunnon *et al.*, 2014). Negligence of screening for strongyloidiasis among individuals with these underlying problems can expose them to the risk of hyperinfection, therefore accurate diagnosis and prompt decision in screening these high-risk patients for *Strongyloides* infection before administrating

immunosuppressant therapy is important and should be given priority (Boulware *et al*., 2007).

1.3.4 Dissemination

Dissemination infection is a stage when larvae is over-proliferated and migrate outside the normal migration route to other organs such as liver, brain, heart, and urinary tract (Kassalik, 2011; Keiser & Nutman, 2004). Patients with disseminated strongyloidiasis typically experience severe clinical presentations such as disseminated intravascular coagulation, meningitis, renal failure or respiratory failure, with sepsis being the most common complication caused by translocation of various bacteria as larvae tend to carry microbial agents with them as invade the bloodstream (Greaves *et al*., 2013; Kassalik, 2011; Mejia & Nutman, 2013,). At this stage, the mortality rate among patients with disseminated infection increase to 85-100% (Mejia & Nutman, 2013). with patients tend to exhibit dreadful clinical presentations such as disseminated intravascular coagulation, meningitis, renal failure or even respiratory failure (Kassalik, 2011). The phrase 'disseminated infection' is often used to refer to migration of larvae to organs outside the range of the pulmonary autoinfective cycle. However according to Keiser and Nutman (2004), this does not necessarily suggest that the disease is more severe at this stage since many cases of hyperinfection resulted in death without the presence of larvae outside the pulmonary autoinfective route. Contrasting to this, a case series was reported in Hong Kong where the dissemination rate approached 100% in disseminated individuals due to late diagnosis (Lam *et al*., 2006).

1.4 Epidemiology of the disease

Strongyloidiasis is a dawning global threat which is underestimated in many countries (Montes *et al.*, 2011), largely due to the low sensitivity of current reference diagnostic tools and the scarcity of in-depth surveys (Olsen *et al.*, 2009). Microscopic examination is most widely used for detection of human intestinal parasite in epidemiological studies even though the sensitivity is low, and misdiagnosis is very likely to happen (Puthiyakunnon *et al*., 2014). Due to intermittent and scanty larval excretion, many chronic infections are missed even with the application of concentration method, which is supposed to improve sensitivity, and as a result, *S. stercoralis* infections are rarely diagnosed, and many places on the global *S. stercoralis* distribution map remain unknown (Puthiyakunnon *et al.*, 2014; Ravi *et al*., 2002). Despite being described as a neglected tropical disease (Beknazarova *et al.*, 2016), the prevalence of this infection is on the rise particularly in underprivileged areas of Southeast Asia, Latin America, Africa, islands of the Caribbean and central, southern and eastern Europe (Puthiyakunnon *et al*., 2014) (Figure 3). The continuous increase in cases is largely attributed to poor practices of personal hygiene, contaminated water supply, weak policy of sanitary measures and poor understanding about the disease among the affected populations (Taylor *et al*., 2014). High prevalence rate of strongyloidiasis in Southeast Asia have also been reported in Cambodia, Lao PDR and Thailand (Puthiyakunnon *et al.*, 2014; Schär *et al*., 2015; Shafik *et al.*, 2018). In Cambodia, a cross sectional study reported 17.4% out of 218 individuals to have been infected by real-time PCR (Schär *et al*., 2013), 45.9% seroprevalence among pregnant women using serological method (Priest *et al*., 2016) and 44.7% of prevalence among rural village in Northern Cambodia by

microscopic method (Khieu *et al.*, 2014). In Thailand, two cross sectional and a case study reported the prevalence from 1.44 % to 9.5% (Angal *et al*., 2015; Azira *et al.*, 2013; Jongwutiwes *et al*., 2014) and two cross sectional study among young population have reported the prevalence as high as 41% in Lao PDR (Laymanivong *et al*., 2016; Vonghachack *et al*., 2015). On the other hand, a retrospective study was conducted in Vietnam and reported 43,000 of blood samples in Medic Center Laboratory, Ho Chi Minh were serologically tested positive for *S. stercoralis*, which mounts up to 7.4 % of seroprevalence (Nguyen *et al*., 2016).

In a more recent finding, Buonfrate *et al*. (2020) summarized the prevalence data from 1990 to 2016 and generate a new predicted value by using spatiotemporal statistical approach which takes into account each studied country's GDP, percentage of rural groups of population, terrain roughness, hygiene practices, annual climatic temperature and rain precipitation per year. This holistic approach was conducted to determine the variable with the highest potential to predict the prevalence of strongyloidiasis, this of which have led to a comprehensive conclusion of 613.9 million global prevalence (8.1%; 95% CI 4.2-12.4%).

In Malaysia, multiple research articles have reported the prevalence of this infection among different target population where the infection ranges from 0.08 % to 11% seroprevalence (Shafik *et al*., 2018). Another recent study estimated the prevalence to be more than 15% (Buonfrate *et al*., 2020). The first study was conducted among Orang Asli community in Kelantan where a single case was reported using formal-ether concentration technique (Rahmah *et al*., 1997). In another study among the Orang Asli community, detection using microscopic method of 54 stool samples resulted in 0% (0/54), however serological examination of the same group revealed a 31.5 % (17/54) of prevalence and followed by a nested PCR

which confirmed 5.6 % (3/17) out of the serologically positive samples to be positive (Ahmad *et al.*, 2013). Meanwhile, another study in Sarawak among patients with gastrointestinal symptoms revealed a high prevalence of 62.3 % (48/77) using pentaplex PCR method (Basuni *et al*., 2011). Further, a recent study was conducted to screen samples from Orang Asli community in Peninsular Malaysia using a few diagnostic methods which were namely direct smear, Koga APC, FES and PCR which then resulted in a relatively high prevalence of 15.8 % (180/1142) (Atroosh *et al*., 2019). In spite the conservative estimates proposing the current prevalence, many researchers believe that this number is awfully underestimated due to the common clinical symptoms this infection has in compared to other illnesses such as diarrhea, vomiting, abdominal pain and is therefore easily misdiagnosed (Rahim *et al*., 2005).

Importantly, strongyloidiasis carries a very arbitrary global burden on account of inaccurate diagnostic methods and due to the fact that there is no reliable gold standard method to rule out the infection (Varatharajalu & Kakuturu, 2016). Fecal tests such as microscopic stool examination tends to underestimate the parasite's infection due to intermittent larval output from infected individuals and low parasite load whereas serology methods such as enzyme-linked immunosorbent assay (ELISA) tends to overestimate the prevalence owing to cross-reactivity problems with other helminthic infections especially in areas co-endemic with strongyloidiasis (Varatharajalu & Kakuturu, 2016). Not to mention most epidemiological studies carried out to screen the prevalence of this infection in many countries still relies on microscopic screening of fecal samples or by concentration and culture methods (Puthiyakunnon *et al*., 2014). This is true to cases in Malaysia as well since routine diagnosis are also performed using previously mentioned methods (Azira *et al*., 2013). This suggests that the actual estimation of this parasite's global burden might

open to a larger number of prevalence.

On another perspective, unclear climate classifications and vague geographical descriptions can also lead to misestimation of the infection (Beknazarova *et al*., 2016). A review article summarized the prevalence of this infection in 39 countries worldwide, accessing the geographical climate zones in correspondence to its socioeconomic condition and deduced that strongyloidiasis is a disease of socioeconomic disadvantage rather than the well-recognized concept that it is a subtropical and a tropical disease (Beknazarova *et al*., 2016). This is due to the case reports shown to have fair amount of occurrences in temperate climates as well, however the logical reasoning to balance this claim is that tropical and subtropical regions are intrinsically linked to poor sanitary practices and low socioeconomic standards (Beknazarova *et al*., 2016). This essential understanding should be the fundamental basis of strategizing a long-term preventive measure and policy to curbing this infection. This is further strengthened with evidential data supporting the notion that communities with low socioeconomic living exhibit higher mortality and morbidity rates in compared to population living with a higher socioeconomic standard (Adler & Ostrove, 1999; Feinstein, 1993).

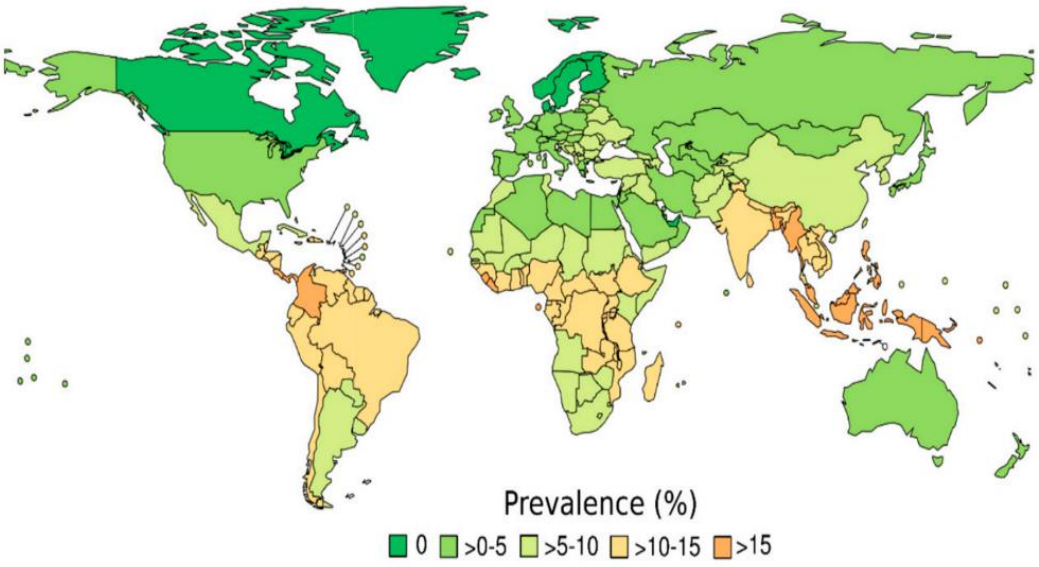

Figure 1.3 Global distribution of *S. stercoralis* infection; Prevalence of the cases reported as predicted in statistical analysis by Buonfrate *et al*., 2020 (Buonfrate *et al*., 2020)

1.5 Mode of transmission

1.5.1 Fecal-oral transmission

S. stercoralis is identified to have been nourished from many reservoirs. This parasite is a soil-transmitted helminth, therefore the transmission of this parasite is illustrated in many scientific literatures to be transdermal, owing to direct contact between an individual's skin and larvae harboring soil (Alabi *et al*., 2017; Arifin *et al*., 2018; Czachor & Patrick Jonas, 2000). Infective larvae enter the host's body percutaneously via skin penetration and escalates through blood circulation up to the lung, where they are coughed up and swallowed before they finally reside in the intestine and resume the infective cycle within the host (Page *et al*., 2018).

1.5.2 Person-to-person transmission

1.5.2 (a) Transmission within household

Cases of person-to-person transmission have also been reported in scholarly literatures, this in which encompasses a case of transmission from an infected individual to another person of close proximity (Czachor & Patrick Jonas, 2000). One of the reported cases was the transmission of infection from a 77-year-old patient having symptoms of aggravated cough, chronic bronchitis, and haemorrhage to his wife; the caregiver who was later diagnosed positive for strongyloidiasis despite of having no symptoms, therefore raising the possibility of an acute person-to-person transmission (Czachor & Patrick Jonas, 2000).

1.5.2 (b) Transmission among homosexual practices

S. stercoralis can also be transmitted through intimate activities involving the perianal region, specifically among homosexual men (Czachor & Patrick Jonas, 2000). A survey to screen enteric parasites was conducted among 180 individuals who attended a sexually transmitted disease (STD) clinic in the United States where three of them were tested positive strongyloidiasis, all of whom practice anal intercourse and oral-anal intercourse (Sorvillo *et al*., 1983). In another case report, 3.9% out of a small group of homosexual men attending an STD clinic were also tested positive of *S. stercoralis* (Philips *et al*., 1981). Case reports of homosexual men developing Kaposi's sarcoma and opportunistic infections linked to suppressed immune defense has given cause for concern that the dissemination stage might be prompted among the male homosexual community and potentially provoke a higher spread of this disease (Haverkovs & Curran, 1982). Another study of a 62-year-old patient has presented a morphologically similar parasitic larvae to *Strongyloides* through colonoscopy, further investigation revealed that patient was in sexual contact with a *Strongyloides*-positive individual (Hechtman *et al*., 2012). Other than that, a recent case series on seven HIV positive men who contracted strongyloidiasis by practicing anal sex, whereby suggesting that skin exposure to fecal material can increase the risk of the parasite's infection (Ross *et al*., 2020).

1.5.2 (c) Transmission from organ donor to organ recipient

Aside from that, cases of person-to-person transmission have also been observed in organ-transplant recipients. Organ-transplant patients are classified as immunocompromised individuals who are at risk of hyperinfection and dissemination of this parasite, a condition which is often fatal (R. M. Genta, 1989). Infection among

the recipients is deduced to be due to reactivation of latent infection or potentially acquire the infection from the organ donor (Abanyie *et al*., 2015; Ben-Youssef *et al*., 2005; Patel *et al*., 2007). An investigation to access *S. stercoralis* transmission via organ transplant cases was conducted by CDC from the year of 2009 to 2013, whereupon seven organ donors were involved (Abanyie *et al*., 2015). In the mentioned cases, none of the donors was screened for the infection prior to the transplanting procedure and consequently, among the seven investigations, 11 out of 20 recipients were showing gastrointestinal and respiratory symptoms, meanwhile out of the 11 recipients, two passed away due to strongyloidiasis complications (Abanyie *et al*., 2015).

1.5.2 (d) Transmission through maternal milk

In more cases of person-to-person transmission, transmammary transmission has also been reported in scientific literature. An early study documented the transmission of infective *S. fuelleborni* larvae passed by a mother to her child during lactation, and this has been the first case ever reported of a parasitic nematode transmitted via human's maternal milk (Rutherford, 1981). Another incident involves the transmission of *S. stercoralis* through mammary glands of a group of female dogs where all pups which suckled from infected female dogs eventually harbored the parasite in their small intestines (Shoop *et al*., 2002). While the evidential reports on human transmammary is lacking, the imposed risk has been well-recognized across multiple literatures and has led to more research on different animal models such as ewes and rats (Dykie et al., 2020; Gross & Thoma-Kress, 2016; Lawrence, 2011). Although the data of transmammary transmission of *S. stercoralis* from a breastfeeding mother to a child is unavailable and most probably still unknown, its

pathological potential should not be neglected (Shoop *et al.*, 2002).

1.5.3 Food-to-consumer transmission

In a current review by White *et al* (2019), *S. stercoralis* can be found from environmental sources such as contaminated food, more specifically vegetables and fruits. A scientific report published a review on 174 scholarly articles worldwide reporting the detection of helminthic parasites on fruits and vegetables, and hereof, 50% of the published findings have identified *S. stercoralis* (Adamu *et al*., 2012; Punsawad *et al*., 2019; White *et al*., 2019). In another study, an evaluation to screen *S. stercoralis* on leafy vegetables in Egypt had been conducted, resulting in 72.5% from the total number of samples carry either eggs, rhabditiform larvae, filariform larvae or the adult free-living *S. stercoralis* females (Amer *et al*., 2020). Likewise, larvae of *S. stercoralis* have also been observed in water samples used to rinse herbs such as pegaga, *kesum* (Vietnamese mint) and water spinach (Zeehaida *et al*., 2011).

The nature of green leafy vegetables is thought to create a conducive habitat for the parasite to subsist due to the rough surface of the vegetables that provides more grip and adhesion (Adamu *et al*., 2012; White *et al*., 2019). Some of the vegetables grow on humid grounds and closely situated to drainage areas, conditions at which enhances the likelihood of harboring the eggs even more (Zeehaida *et al*., 2011). Thus, the group of individuals who are highly involved in handling these commodities are at the frontline of the infection from this particular source, owing to constant exposure they face on a daily basis (Zeehaida *et al*., 2011). This too carries the possibility of transmission to consumers if not treated wisely (Zeehaida *et al*., 2011).

1.6 Clinical significance

1.6.1 Association with corticosteroids

The occurrence of strongyloidiasis has been surging worldwide where many scientific literatures have described the most susceptible groups to be patients of suppressed immunity, linked with malnutrition, tumors, and organ-transplant patients (Mirdha, 2009). Patients administrated with corticosteroid therapy could trigger an overwhelming autoinfection cycle which then spur the larvae to migrate beyond the usual migratory tract, invading all organs and tissues (Olsen *et al.*, 2009). One of the most widely used immunosuppressive drugs administrated to patients is the glucocorticoids which are notoriously associated to have been responsible in aggravating chronic strongyloidiasis to hyperinfection, increasing the risk two to three- fold (Keiser & Nutman, 2004) (Mirdha, 2009). Several lines of evidence suggest that glucocorticoids trigger hyperinfection as a result of chemical activity that suppress eosinophilia and lymphocyte activation (Keiser & Nutman, 2004). Additionally, other immunosuppressants such as azathioprine, cyclophosphamide, antithymocyte globulin, adriamycin, doxorubicin, melphalan have also been described to be linked to strongyloidiasis (Mejia & Nutman, 2013).

On another view, some authors also described that the direct effect of the drug itself on parasites further accelerates the transformation of the rhabditiform larvae stages into infective filariforms (R. M. Genta, 1992). This is due to the discovery of glucocorticoids metabolite in serum and urine samples of patients which resemble an ecdysteroid-like substance; a steroid molting hormone, hence further augmenting the speculation of ecsysteroids in transmitting molting signals (R. M. Genta, 1992; Khadka *et al.*, 2018). Ultimately, this then triggers the rhabditiform larvae to mature into filariform larvae which resultantly exacerbates the infection (Khadka *et al.*,

2018).

1.6.2 Association with viral infections

This group of individuals is commonly observed in patients infected with HTLV-1 virus and HIV infection (Keiser & Nutman, 2004; Mirdha, 2009; Mun *et al*., 2013). While both glucocorticoids and HTLV-1 infection are almost identical as both are cell-mediated immune suppressants, the two differ in their immunosuppressive activities (Siegel & Simon, 2012). Other than being related to T-cell lymphoma, HTLV-1 has also been linked to *S. stercoralis* infection since it suppresses the host's immune system, prompting the infection to take place (Mendes *et al*., 2017).

HTLV-1, or the human T cell lymphotrophic virus type 1 is a viral infection which infects human's T cells and subsequently induces a voluntary lymphocyte proliferation alongside with secretion of type 1 cytokines (Mirdha, 2009). A study conducted in Japan reported that the risk of getting hyperinfection is twice higher among HTLV-1 patients when compared to their control group (Hirata *et al*., 2006). Patients coinfected with strongyloidiasis and HTLV-1 usually present a modified immunological response towards parasitic antigens where their Th1 response is induced to be stronger, but the Th2 response is weakened, this ensues low secretions of cytokines which are important in regulating immune response (Mirdha, 2009). Consequently, this hinders eosinophil count and parasite killing activities (Satoh *et al*., 2002), hence these patients were observed to produce less polyclonal and IgE which are specific to the antigen (Keiser & Nutman, 2004).

In contrast to HTLV-1 infection which can be considered as one of the strongest risk factors (Keiser & Nutman, 2004), it is rather the opposite for cases involving patients with acquired immunodeficiency syndrome (AIDS). Although *S. stercoralis* is recognized to be an opportunistic infection among individuals affected