ISOLATION, CHARACTERIZATION, AND PRODUCTION OF RECOMBINANT MONOCLONAL ANTIBODIES AGAINST rNIE FOR DEVELOPMENT OF A *STRONGYLOIDES* ANTIGEN DETECTION ASSAY

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by

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ABTS	2,2'-Azino-d-[3-ethylbenthiazoline sulfonate]
Ab	antibody
ADCC	antibody dependent cellular cytotoxicity
APC	antigen presenting cells
APS	ammonium persulfate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
CAI	codon adaptation index
cDNA	complementary DNA
CDR	complementary determining region
COV	cut-off value
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOT	diffractive optics technology
E. coli	*Escherichia coli*
ECP	eosinophil cationic protein
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FECT	formalin-ether concentration technique
FES	formalin-ether sedimentation
GPIAT	gelatin particles indirect agglutination test HC heavy chain
HC	heavy chain

His	histidine
HIV	human immunodeficiency virus
HRM	High Resolution Melting
HRP	horse-radish peroxidase
HTLV-1	Human T-cell lymphotropic virus
i.e.	id est (that is)
IFAT	immunofluorescence antibody test ILinterleukin
IMAC	immobilised metal affinity chromatography (IMAC)
INFORMM	Institute for Research in Molecular Medicine
IPTG	isopropyl-beta-D-thiogalactopyranoside
Kb	kilo base pair
KC	keratinocyte chemoattractant
K_D	equilibrium dissociation constant
kDa	kilo Dalton
LC	light chain
LIPS	luciferase immunoprecipitation system
LAMP	loop-mediated isothermal amplification
MAb	monoclonal antibody
MBP	major basic protein
MIP-2	macrophage-inflammatory protein-2
MPO	myeloperoxidase
MW	molecular weight
NC	nitrocellulose membrane
Ni-NTA	nickel-nitrilotriacetic acid
NTD	neglected tropical diseases

OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	Phycoerythrin
PEG	Polyethylene glycol
Pfu	plaque forming unit
p*I*	isoelectric point
PTM	Milk powder in PBST (Blocking buffer)
RC DC	reducing agent detergent compatible
RE	Restriction enzyme
RFLP	restriction fragment lengths polymorphism
rMAb	Recombinant monoclonal antibody
RUC	*Renilla* luciferase
scFv	single chain fragment variable
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
SEA	Southeast Asia
STH	soil-transmitted helminths
TB	terrific broth
TBS	tris buffered saline
TEMED	tetramethylethylenediamine
TLR-4	toll-like receptor 4
UV	ultraviolet
VDJ	variability, diversity and joining
WHO	World Health Organisation

PENGASINGAN, PENCIRIAN, DAN PENGHASILAN ANTIBODI MONOKLON TERHADAP rNIE TERHADAP PEMBANGUNAN ASAI PENGESANAN ANTIGEN *STRONGYLOIDES*

ABSTRAK

Strongyloides stercoralis adalah helmin tularan tanah yang menyebabkan penyakit strongyloidiasis, dan dianggarkan menjangkiti lebih 600 juta manusia di dunia. Jangkitan kronik tanpa gejala pada orang yang mengalami masalah immunokompromi boleh menyebabkan hiperinfeksi yang mungkin membawa maut. Serodiagnosis jangkitan melalui pengesanan antibodi IgG berkemungkinan menunjukkan reaktiviti silang dengan jangkitan helmin lain. Ujian pengesanan antigen ialah satu kaedah pengesanan langsung yang dapat membantu diagnosis, dan juga berguna untuk pemantauan pesakit pasca-rawatan. Kajian ini menggunakan teknologi paparan faj untuk menghasilkan antibodi monoklon rekombinan (rMAb) terhadap protein rekombinan NIE (rNIE) dan seterusnya membangunkan ujian pengesanan antigen *Strongyloides*. rNIE adalah protein telah terbukti berguna bagi diagnosis penyakit strongyloidiasis. rNIE diekspresi dan dipurifikasi, kemudian digunakan untuk mendapatkan calon rMAb melalui teknik *biopannin*g ke atas perpustakaan paparan faj imun. Ia berjaya mengasingkan 104 klon positif-ELISA, dan analisis menunjukkan bahawa 30 klon mempunyai rantai ringan dan rantai berat yang panjangnya lengkap. Empat famili gen unik dikenalpasti, iaitu IgHV3-LV6 (86.66%), IgHV1-LV3 (3.33%), IgHV5-KV3 (3.33%), dan IgHV3-LV3 (6.66%). Secara rawak, satu klon daripada setiap famili gen telah dipilih untuk kajian lanjutan, iaitu, (a) rMAb5 mewakili IgHV1-LV3, (b) rMAb6 mewakili IgHV3-LV6, (c) rMAb14 mewakili IgHV5-KV3, dan (d) rMAb23 mewakili IgHV3-LV3. Jujukan gen rMAb daripada

vektor paparan faj diklonkan ke dalam vektor ekspresi pET51b + dan ditransformasi ke dalam sel host *Escherichia coli* Shuffle T7 Express. Ekspresi dan purifikasi protein rMAb dilakukan, diikuti dengan kajian pencirian. Blot Western mengesahkan kehadiran tag Strep dalam vektor rMAb, dan Blot Western Antigen-Antibody (Ag-Ab) menunjukkan keempat-empat rMAb terikat secara khusus pada rNIE. Kekuatan pengikatan setiap rMAb pada rNIE melalui ELISA titrasi menunjukkan bahawa rMAb5 dan rMAb14 mempamerkan pengikatan pada kepekatan terendah (0.156 µg/mL), diikuti oleh rMAb23 (0.3125 µg/mL), dan rMAb6 (1.25 µg/mL). ELISA kereaktifan silang (*cross-reactivity ELISA*) mengkaji sifat pengikatan rMAbs terhadap antigen yang sama dan hasilnya menunjukkan bahawa keempat-empat rMAbs mengenalpasti epitop yang sama. Blot Western dan ELISA menggunakan antigen NIE natif daripada larva lisat *Strongyloides* menunjukkan adanya pengikatan dengan keempat-empat rMAbs. Afiniti pengikatan keempat-empat rMAbs terhadap rNIE menggunakan tenik resonans plasmon permukaan (SPR) menunjukkan bahawa rMAb23 mempunyai pengikatan terkuat terhadap rNIE. Seterusnya rMAb23 digunakan untuk pembangunan ELISA pengesanan antigen *Strongyloides* bagi diagnosis penyakit strongyloidiasis (SsAg-ELISA). ELISA yang dibangunkan menunjukkan nilai 100% kepekaan (*sensitivity*) dan kekhususan (*specificity*) diagnostik. SsAg-ELISA juga berkemungkinan dapat membezakan jangkitan awal/akut dan kronik. Sebagai kesimpulan, rNIE yang tulen telah dihasilkan dan berjaya digunakan untuk proses *biopanning* ke atas perpustakaan paparan faj helmin imun. Empat famili gen antibodi monoklonal spesifik terhadap *Strongyloides* diasingkan. Empat rMAb dicirikan, dan pengikatannya pada rNIE dan antigen NIE natif disahkan. rMAb23 telah digunakan untuk pembangunan ELISA pengesanan

antigen yang menunjukkan nilai 100% kepekaan dan kekhususan diagnostik bagi mengesan jangkitan oleh *Strongyloides*.

ISOLATION, CHARACTERIZATION, AND PRODUCTION OF RECOMBINANT MONOCLONAL ANTIBODIES AGAINST rNIE FOR DEVELOPMENT OF A *STRONGYLOIDES* ANTIGEN DETECTION ASSAY

ABSTRACT

Strongyloides stercoralis is a soil-transmitted helminth that causes strongyloidiasis. It is estimated to infect more than 600 million people worldwide. Asymptomatic chronic infections in immunocompromised people can lead to fatal hyperinfection. Serodiagnosis by detecting specific IgG antibodies can be challenging due to potential cross-reactivity with infections by other parasites. An antigen detection assay, a direct detection method, can help the diagnosis and is useful for post-treatment follow-up. This study used phage display technology to produce recombinant monoclonal antibodies (rMAb) against NIE recombinant protein (rNIE) and develop a *Strongyloides* antigen detection test. rNIE is an established protein for the diagnosis of strongyloidiasis. rNIE was expressed, purified, and then used to select rMAb candidates via biopanning of an immune helminth phage display library. It isolated of 104 ELISA-positive clones and sequence analysis showed that 30 clones had full-length light and heavy chains. Four unique gene families were identified, i.e., IgHV3-LV6 (86.66%), IgHV1-LV3 (3.33%), IgHV5-KV3 (3.33%), and IgHV3-LV3 (6.66%). Randomly, one representative clone from each gene family was selected for further studies, i.e., (a) rMAb5 representing IgHV1-LV3, (b) rMAb6 representing IgHV3-LV6, (c) rMAb14 representing IgHV5-KV3, and (d) rMAb23 representing IgHV3-LV3. The rMAb gene sequences from the phage display vector were subcloned into the pET51b+ expression vector and transformed into *Escherichia coli* Shuffle T7 Express host cell. The expression and purification of the rMAb proteins were carried

out, followed by characterization studies. Direct Western blot confirmed the presence of the Strep-tag in the rMAb vectors, and Antigen-Antibody (Ag-Ab) Western blot showed that all four rMAbs were specifically bound to rNIE. The binding strength of each rMAb to rNIE, determined by titration ELISA, showed that rMAb5 and rMAb14 were able to bind at the lowest concentration (0.156 µg/mL), followed by rMAb23 (0.3125 µg/mL), and rMAb6 (1.25 µg/mL). Cross-reactivity ELISA was performed to study the binding properties of the rMAbs against the same antigen, and the results indicated that all four rMAbs recognized the same epitope. Western blot and ELISA using native NIE antigen from *Strongyloides* larval lysate showed binding with all four rMAbs. The binding propensity of the four rMAbs to rNIE, determined using surface plasmon resonance (SPR), showed that rMAb23 had the strongest binding affinity. Subsequently, rMAb23 was used to develop an antigen detection ELISA for strongyloidiasis (SsAg-ELISA). The ELISA showed 100% diagnostic sensitivity and specificity. The developed assay may potentially distinguish early/acute and chronic infections. In conclusion, purified rNIE was produced and successfully used for the biopanning of an immune helminth phage display library. Four distinct gene families of *Strongyloides* specific-monoclonal antibodies were isolated, characterized, and their bindings to rNIE and native NIE antigen were validated. rMAb23 was used to develop an antigen detection ELISA that showed a 100% diagnostic sensitivity and specificity for detecting *Strongyloides* infection.

CHAPTER 1 INTRODUCTION

1.1 Strongyloidiasis: An overview

Human strongyloidiasis is a soil-transmitted helminth mainly caused by the nematode *Strongyloides stercoralis*. This disease can sometimes be caused by *S. kellyi* and *S. fuelleborni*. Strongyloidiasis is among the twenty neglected tropical diseases and disease groups (NTDs) identified by the World Health Organisation (WHO). It is estimated that 613.9 million people are infected globally, with endemicity varying by continents and countries (Bisoffi et al., 2013). Nevertheless, due to the difficulty in quantifying the infection and the complexity of the diagnostic methods, there is a distinct lack of data as to true infection rates and spread (Montes et al, 2010).

In immunocompetent individuals, *Strongyloides* infection usually causes long-term and asymptomatic infections that are difficult to diagnose clinically. However, *S. stercoralis* holds great significance in terms of severe morbidity and a higher than normal mortality in immunocompromised people in the form of hyperinfection and disseminated infection (Keiser et al., 2004; Kandi, 2017).

1.2 Background of *S. stercoralis*

1.2.1 Discovery of *S. stercoralis*

S. stercoralis was initially identified in the year 1876 by French soldiers who discovered it in Cochin-China and it was called as *Anguillula stercoralis* (Lindo et al., 2001). It was in 1902 that Stiles and Hassall and the International Commission on Zoological Nomenclature accepted the correct name of *Strongyloides stercoralis* (Lindo et al., 2001). It was Looss in the year 1904 that infected himself with the

filariform larvae to show the process of the movement of the parasite in the human body. Sixty four days after being exposed, the larvae were still found in his faeces (Sandground, 1925). In 1914, Friedrich Fülleborn discovered the autoinfection cycle (Cox, 2002). The disseminated infection in immunosuppressed patients was discovered in the 1940s in a study on infections among war prisoners who had acquired the infection in the Far East (Gill et al., 1979). The complete life cycle, pathology, and clinical features of the disease were detailed in the 1930s (Schär et al., 2013).

1.2.2 Taxonomy of *S. stercoralis*

S. stercoralis is classified by nomenclature under the Class of Nematoda, Order *Rhabdiasidea,* Family *Strongyloididae,* and Genus *Strongyloides.* Nematodes are considered to be non-segmented worms with a filiform body, and the vast majority of the species (99%) are extremely diverse and exist across the water bodies and earthy environments (Decraemer, 2019). More than 50% of nematode species are parasitic and to date, 81.6 million cases are documented (Zhang, 2013; Larsen et al., 2017). Over 52 species of the genus *Strongyloides* are gastrointestinal obligate parasites of reptiles, birds, amphibians, and mammals.

Strongyloides infectious species are *S. stercoralis, S. fuelleborni, S. myopotami, S. myopotami,* and *S. procynosis* (Ashford, 1989; Goncalves et al., 2007). In humans, strongyloidiasis is caused by *S. stercoralis, S. fuelleborni*, and *S. fuelleborni kellyi* (Requena-Méndez et al., 2013; Taylor et al., 2014). *S. stercoralis* mostly affects humans while *S. fuelleborni* mostly infects non-human primates and sometimes causes sporadic zoonotic disease. Meanwhile, *S. fuelleborni kellyi* has only been observed in Papua New Guinea where the symptoms are exhibited in babies as "swollen belly" syndrome (Ashford et al. 1992).

1.2.3 Morphology of *S. stercoralis*

It has been found that *S. stercoralis* has two distinct life cycles, one is a free living in the environment and the other is parasitic in the host. Both genders of parasite survive in the bowel, however the females alone reach adulthood and are able to reproduce parthogenetically and start their parasitic stage in humans while the male disappears after the release of the eggs (Cook et al., 2008). The eggs are oval, thin-shelled entities that are partially embryonated during the 2-8 cell stage of establishment, measuring 50-58 µm in length and 30-34µm in breadth. Free-living first stage (L1) and second stage (L2) larvae can grow to 350 µm long and have a rhabditiform pharynx with a muscular oesophagus for consuming particulate materials. The third-stage infective larvae (L3) have a filariform pharynx with a long, fine oesophagus for collecting fluids through host tissue penetration and can grow up to 600 µm in length. The larvae are encased by a closed mouth and a pointed notched tail and do not eat in the soil. Parthenogenetic females range in length from 2-3 mm and are distinguished by a long filariform pharynx throat that is one-third the length of the body and a blunt pointed tail. Male and female free-living worms have a rhabditiform pharynx, which is smaller and measures up to 1 mm in length. Male worm contains two spicules and a gubernaculum, which has a curved ventrally pointed tail. Female has a large vulva, which is placed in the centre of the body (Schad et al., 1993; Ashton et al., 1995; Lindo & Lee, 2001).

1.2.4 Life Cycle of *S. stercoralis*

S. stercoralis is a unique nematode which shifts between parasitic and free-living life cycles alternately. The free-living cycle is divided into two types, i.e., homogenic and heterogenic. In the homogenic cycle, the L1 rhabditiform larvae leaves

the human host and moults twice to become an infective filariform L3 for up to two weeks until it gets a new host to infect (Grove, 1996). In the heterogenic cycle, the L1 exits the host's body through faeces and undergoes moulting four times to become a free living adult worm (Schad, 1989). They have the capacity to survive for 2-4 days outside a host body and last for a single generation (Conway et al., 1995). They reproduce by mating and release eggs that hatch to become L1, then changes into L3 which just have one target, namely to find a human host to infect (Yamada et al., 1991).

The parasitic cycle begins with the filariform infective larvae penetrating the skin into the circulatory system and migrate to the lungs and alveolar spaces. The larvae then ascends the trachea where they end up being swallowed and migrate to the small intestine (Woll et al., 2013). However, L3 larvae can migrate to the intestine via alternate routes such as through abdominal viscera or connective tissue (Greiner et al., 2008). The larvae then moult twice to transform into mature parasitic females that digs into the intestinal epithelium surface of the gut and produce eggs through parthenogenesis in the mucosa of the intestine where the egg grows to L1 that travels to the intestinal lumen. The overall life cycle is portrayed in Figure 1.1.

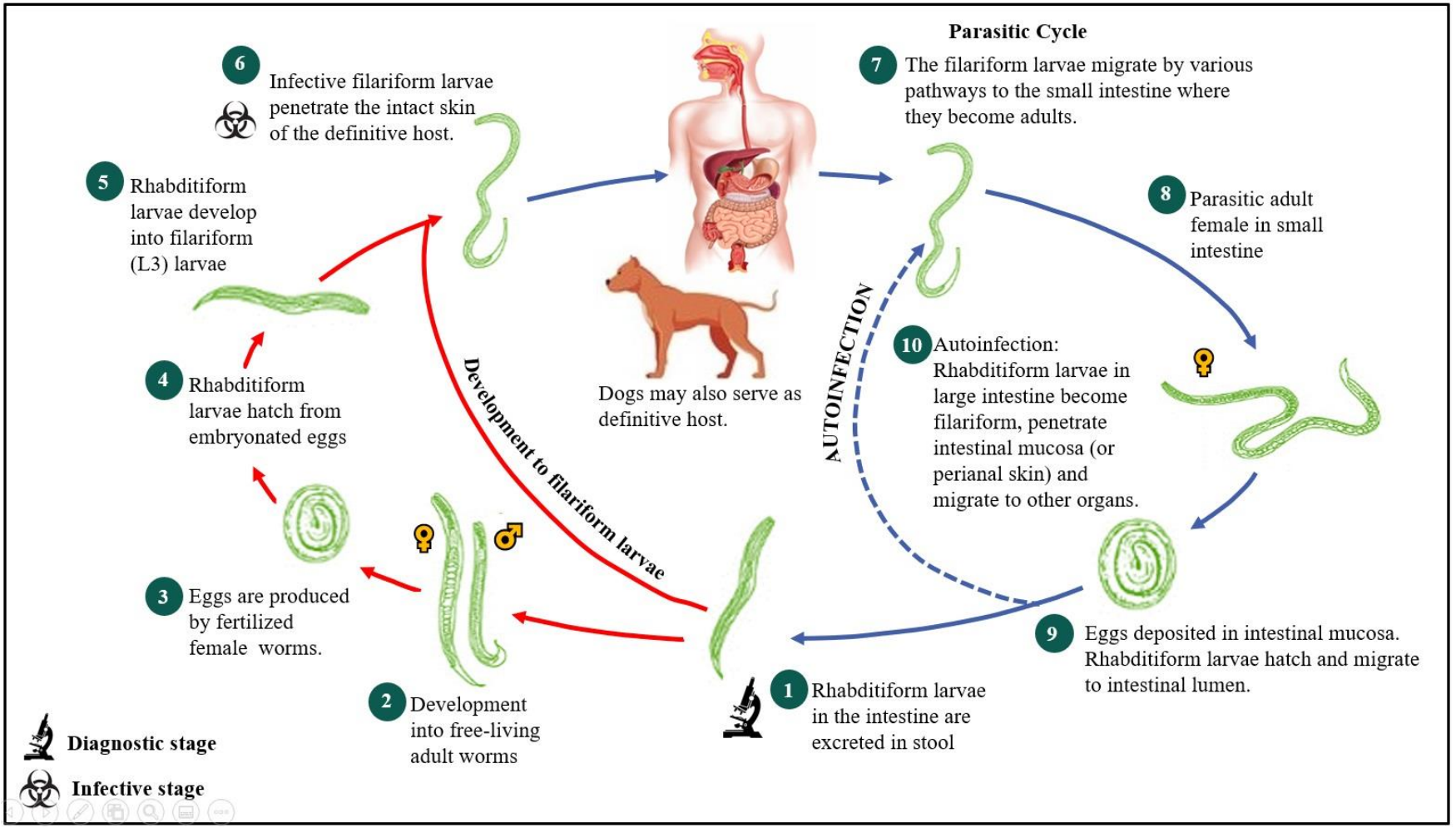

Figure 1.1 The life cycle of *S. stercoralis*. Steps 1-10 indicate different stages of the life cycle of *S. stercoralis.*

1.2.4(a) Autoinfection cycle of *S. stercoralis*

Parasitic *S. stercoralis* females produce offspring by parthenogenesis. The parasite can then reproduce and continue its life cycle inside the same host, a phenomenon known as 'autoinfection', and unique to *S. stercoralis*. After the larvae hatched from the ova, they can exit from the host with the faecal matter to start a free-living cycle. The larvae can also cause an internal autoinfection cycle, whereby the larvae moult twice into L3 and penetrate into the intestinal mucosa. The L1 can also migrate to the perianal area, transforms into L3, then penetrate the skin, causing external autoinfection (Mansfield et al., 1996; Viney et al., 2007). Thus the parasite can stay in the host for decades (Leighton et al., 1990).

The autoinfection cycle is controlled and regulated by the host's immunological state (Lindo & Lee, 2001). With a fully functioning immune system, the chronic autoinfection has been recorded to survive for up to 75 years (Prendki et al., 2011). In immunocompromised patients, the low-level autoinfection can transform into hyperinfection, in which large numbers of larvae penetrate inside the gut, travelling through the lungs and going back into the intestine. Since the larvae carry the gut bacteria on its body, the hyperinfection can lead to secondary bacteraemia. The larvae eventually spreads into other organs leading to disseminated strongyloidiasis (Clark, 2020).

1.3 Transmission of *S. stercoralis*

Humans usually acquire the infection when the larvae penetrate the skin and travel in the body. The most common way is through exposure with contaminated soil that allows the filariform larvae to initiate contact with skin (Freedman, 1991). Looss infected himself with filariform larvae to proof transmission by skin penetration. After

a period of 64 days post-infection, he found the larvae in his faecal matter (Sandground, 1925). The risks for getting this infection increases with exposure to soil that is polluted with human faecal matter which provides the perfect environment for the survival of *S. stercoralis* (Krolewiecki & Nutman, 2019). Health care providers, coal mine workers, gardeners, and farmers are at high risk of acquiring the infection (Puthiyakunnon et al., 2014). Furthermore, humans can also get *Strongyloides* infection through food or water contaminated with infectious larvae. The oral ingestion of larvae was shown by Wilms, he detected the faeces and larvae in after just 17 days (Wilms, 1897; Grove, 1989). The larvae ingested directly in contaminated food or water can escape lung migration and move directly to the small intestine (Greiner et al., 2008). *S. stercoralis* larvae have been revealed in vegetables cultivated in contaminated soil, therefore individuals who sell or prepare these vegetables are also at risk of acquiring this infection (Zeehaida et al., 2011).

Direct person-to-person transmission is not common, although a case has been reported of a woman who acquired the infection from her infected husband, and reports of sexual transmission in homosexual males (Sorvillo et al., 1983; Czachor et al., 2000; Ross et al., 2020). Transmission through transmammary route has been only observed in dogs that were infected at late gestation period and during lactation (Shoop et al., 2002; Baker, 2007).

1.4 Epidemiology of *S. stercoralis*

S. stercoralis has been discovered in all continents apart from Antarctica (CDC, 2020b). The helminth is most typically found in the subtropics, tropics, and warm temperate zones. The global estimate is approximately 100-690 million people infected (Fleitas et al., 2020; Buonfrate et al., 2020). The endemicity of

strongyloidiasis varies from country to country and also among continents depending on the population studied, climate, socioeconomic status, and the method of diagnosis (Beknazarova et al., 2016). Majority of cases are found in rural populations with people living in poverty, immigrants, former war veterans, travellers, immunocompromised populations, or groups occupationally outed to soil (Beknazarova, 2019). The incidence is underreported due to insufficient diagnostic procedures, limited sensitivity of existing tests, and symptoms similar to other helminth infections (Beknazarova et al., 2016). Furthermore, the majority of the research were conducted on people who might be at risk group or in hospitals, making it difficult to extrapolate the findings to the overall population (Abd Majid et al., 2018).

Published reports on prevalence among immigrants showed that 90.4% were from Sub-Saharan Africa and 64.7% from South East Asian countries; among the latter 42% were from Cambodia and 24% from Laos (Gyorkos et al., 1990; De Silva et al., 2002; Caruana et al., 2006; Cabezas-Fernández et al., 2015). In the United Kingdom, former World War II Far East prisoners showed a prevalence rate of 12% (Gill et al., 2004). In Canada, the prevalence rates ranged from 9% to 77%, mostly from Southeast Asian immigrants (Thompson et al., 2015).

In random populations, the highest prevalence reported was 44.7% in Northern Cambodia, followed by 29.2% in Brazil, 25% in Southeast Asia, 12.4% in Spain, 11.7% in China, 11.6% in Northern Ghana, and 8.5% in India (Gyorkos et al., 1990; Roman-Sanchez et al., 2003; Yelifari et al., 2005; Steinmann et al., 2007; Devi et al., 2011; Paula et al., 2011; Khieu et al., 2014).

In community-based prevalence studies, the highest prevalence of 31.5% was reported in the Orang Asli (aborigine) community of Malaysia (Ahmad et al., 2013), 21% in aboriginal communities in Northern Australia (Kearns et al., 2017), and 27.5%

in children of aboriginal communities in Queensland Australia (Prociv et al., 1993). In hospital-based prevalence studies, 39% prevalence was reported in Sarawak Hospital, Malaysia (Basuni et al., 2011), 33% in Royal Darwin Hospital, Australia (Fisher et al., 1993), 29.4% at the Instituto de Investigaciones de Enfermedades Tropicales, Orán, Argentina (Krolewiecki et al., 2010), 11.2% in nine hospital in India (Schär et al., 2013), and 10.8% in the Campinas City region, Brazil (Rossi et al., 1993).

The high prevalence rates of strongyloidiasis in Southeast Asian countries is associated with the ecological, climatic, and socioeconomic conditions that support the spread of and survival of *S. stercoralis*. Various diagnostic methods were used for screening; hence the reported prevalence rates showed heterogeneous results suggesting that a reliable estimate of prevalence for Southeast Asia is challenging. There may be a possibility of underreporting of cases especially in places that may suggest higher transmission rates of *S. stercoralis* (Schar et al., 2013).

S. stercoralis prevalence has been extensively studied in Thailand with a range of 0.1% to 47.5% in local rural populations, the wide range of prevalence rates could be attributed to the varied approaches used. The studies gave excellent illustrations of the problem of estimating infection rates (Prociv & Luke, 1993; Abd Majid et al., 2018). In Cambodia, the prevalence rate of 45.9% was reported among women aged 15–39 year (Priest et al., 2016), and 17.4% among school children (Schär et al., 2013). In two cross-sectional studies, 41% of the general population in Laos was infected with *S. stercoralis* (Vonghachack et al., 2015; Laymanivong et al., 2016). A retrospective study found 7.4% seroprevalence of strongyloidiasis in the southwest region of Vietnam (Nguyen et al. 2016). In Indonesia, the rate of prevalence of 0.8% to 5.4% was found based on seven screening studies (Schär et al., 2016). A study carried out in the general population of six provinces of the Philippines found a prevalence rate of

1.18% (Cabrera, 1981). There are no published reports on the prevalence rate of strongyloidiasis in countries like Brunei, Singapore, and Myanmar (Schär et al., 2016; Abd Majid et al., 2018).

In Malaysia, the prevalence data of *S. stercoralis* infection is still limited and most of the studies conducted were based on community and hospital populations. A prevalence rate of 1.2 % was reported among children in an aboriginal community in Kelantan using the microscopic technique (Rahmah et al., 1997). Cross-sectional studies of Orang Asli primary school children in six states of Peninsular Malaysia showed 15.8% prevalence of *S. stercoralis* infection using various diagnostic techniques, including direct smear, formalin-ether sedimentation, Koga agar plate culture (APC), and polymerase chain reaction (PCR) (Al-Mekhlafi et al., 2019). In addition, the seropositive rate of 31.5% was found in the indigenous communities in Selangor, West Malaysia (Ahmad et al., 2013) and 11% in indigenous communities in Borneo Island, East Malaysia (Ngui et al., 2016). Meanwhile, a hospital-based study conducted at two district hospitals in Sarawak, Malaysia reported a 39% prevalence rate by a real-time PCR technique (Basuni et al., 2011). Also, a study carried out among migrant workers in Malaysia showed 35.8% prevalence (Sahimin et al., 2019).

Patients who are administered systemic corticosteroids, for example, transplant recipients, are at high risk of strongyloidiasis. One study reported that there were 54 cases of strongyloidiasis among renal transplant patients, 9 occurrences in non-renal solid organ transplants and 7 cases in hematopoietic stem cell transplants (HSCT) (Snydman et al., 2009). Montes et al., (2009) found that *Strongyloides* infections were also documented in patients with cancers, human immunodeficiency virus (HIV), undernourishment, chronic obstructive pulmonary disease (COPD), human T-lymphotropic virus-1 (HTLV-1) associated myelopathy, chronic renal failure and

diabetes mellitus. In Brazil, 13% prevalence rate of strongyloidiasis was found in cancer patients. Among them, 27% had acute leukemia, 36% lymphoma, 9% multiple myeloma, 9% chronic lymphocytic leukemia, and 18% with myeloproliferative disease (Schaffel et al., 2001). In Malaysia, a seroprevalence of 3.1% was identified among cancer patients in a Kelantan hospital (Zueter et al., 2014).

It has been observed that regions or countries that adopted multiple diagnostic approaches or had screened a large number of samples reported high rates of prevalence of strongyloidiasis. Meanwhile, a low prevalence rate was reported in areas where diagnostic methods with low sensitivity were used. This shows that global prevalence of strongyloidiasis may be underestimated. Thus, the development of sensitive diagnostic methods for *S. stercoralis* infection is essential for detecting the disease as well as for epidemiological studies.

1.5 Clinical Manifestations of Strongyloidiasis

Majority of strongyloidiasis patients exhibit symptoms which are uncomplicated and can survive undiagnosed for decades. When a patient is symptomatic, the clinical manifestation can range from mild uncomplicated strongyloidiasis to hyperinfection syndrome and disseminated strongyloidiasis. In uncomplicated strongyloidiasis, many patients exhibit uncharacterised clinical symptoms. Disseminated strongyloidiasis and hyperinfection can be lethal in immunocompromised people due to uncontrolled parasite growth and, in the former, larvae dissemination to all internal organs (Keiser & Nutman, 2004; Mejia et al., 2012).

1.5.1 Acute Strongyloidiasis

The clinical manifestation of acute strongyloidiasis is exhibited in the pre-patent period (2-4 weeks) which starts from the penetration of infective larvae until new larvae production by adult females (Freedman, 1991). The signs are linked to the larval migration path from the point of introduction to where the adults grow and start producing new larvae. A few minutes after penetrating the skin, the site of larvae entry itches and within 24 hours, there is skin irritation followed occasionally by localised edema or urticaria, pruritic rash, and erythematous macules (Meyers et al., 2000; Kling et al., 2016; Alabi et al., 2017). After a week, the larvae migrating via the lungs may irk the throat, leading to cough and symptoms similar to Löffler's disease such as wheezing, shortness of breath, and dyspnea (Mahmoud, 1996). The larvae reach the intestine three weeks after infection, and gastrointestinal symptoms begin to develop (Keiser & Nutman, 2004). Acute gastrointestinal symptoms are indigestion, bloating, abdominal pain, anorexia, nausea, discomfort, watery diarrhea, and malabsorption (Meyers et al., 2000; Krolewiecki & Nutman, 2019).

1.5.2 Chronic Strongyloidiasis

Chronic strongyloidiasis is most often asymptomatic or mildly symptomatic with non-specific symptoms (Grove, 1980; Krolewiecki & Nutman, 2019). The gastrointestinal tract and the skin are the most commonly affected areas when symptoms appear. Diarrhoea, constipation, cramping in the lower abdomen with discomfort, pruritis ani, occasional weight loss, and sporadic vomiting are all chronic gastrointestinal symptoms. Mucosal injury occurs in the large intestine on rare occasions, resulting in eosinophilic colitis, pseudopolyposis and ulcerative colitis, usually in older patients (Carp et al., 1987; Gutierrez et al., 1996; Al Samman et al.,

1999). Dermatological manifestation include non-specific urticarial rash and serpiginous migratory larva currens which moves at a rate of 5–15 cm/hour through the subcutaneous tissue of thighs, buttocks, and lower trunk (Byard, 2019). The larva currens were observed in a majority of World War II prisoners (30%–92%) who acquired strongyloidiasis in the Southeast Asian region (Grove, 1980; Gill et al., 2004). Respiratory symptoms were also significantly manifested in this phase (Grove, 1980; Grove, 1996). Unusual reported manifestations of chronic strongyloidiasis include massive upper gastrointestinal bleed (Jaka et al., 2013), hepatic lesion (Gulbas et al., 2004), nephrotic syndrome (Hsieh et al., 2006), ascites (Hong et al., 2004), asthma (Dunlap et al., 1984), and arthritis are found in HLA-B27-positive individuals (Richter et al., 2006). Due to autoinfection, these conditions can last for decades in immunocompetent people.

1.5.3 Severe Manifestations of Strongyloidiasis

Strongyloidiasis can turn into hyperinfection syndrome if the immune system is not fully functional, with a mortality rate of up to 85%–100% (Lam et al., 2006; Mejia & Nutman, 2012). Immunosuppressed patients have shown huge increase in worm burden, which may lead to hyperinfection, and subsequently disseminate outside their typical migration route, i.e., pulmonary and gastrointestinal tract. A study of 244 case reports was conducted (73 cases of dissemination and 171 cases of hyperinfection) and documented that 67% (164/244) of the cases were associated with corticosteroid use, 15% (38/244) with HIV (3% having concomitant corticosteroid use), 11.5% (28/244) with solid organ transplant recipients (10% having concomitant corticosteroid use), and 10 % (24/244) with HTLV-1 disease (Buonfrate et al., 2013; Page et al., 2018). In immunocompromised conditions, the reproduction of *S.*

stercoralis is overwhelming which leads to a massive invasion of larvae in the bowels and lungs. The number of larvae increases in non-disseminated hyperinfection, but they are limited to the organs involved in the typical migration route. The high mortality is often due to the enteric bacteria (*Escherichia coli*, *Klebsiella pneumonia*, *Streptococcus fecalis*, *Streptococcus bovis* or *Enterobacter sp)* and yeast travelling with filariform larvae. They may gain systemic accesses through intestinal ulcers and affect other organs system causing bacteremia, meningitis, peritonitis, and endocarditis (Igra-Siegman et al., 1981; Keiser & Nutman, 2004; Krolewiecki & Nutman, 2019; Clark et al., 2020). During hyperinfection, the patients may experience ileus and small bowel obstruction, with diffuse tenderness and hypoactive bowel sounds (Clark, 2020; Yazdanpanah et al., 2020). Continuous autoinfection cycle with immunosuppression increases the parasite load which may lead to the involvement of multiple organs. The presence of larvae in any organ other than the gastrointestinal tract and respiratory defines disseminated strongyloidiasis (Buonfrate et al., 2013). The liver, skin, gallbladder, kidneys, pancreas, ovaries, diaphragm, skeletal muscle, mesenteric lymph nodes, brain, and heart can be affected by disseminated strongyloidiasis (Nutman, 2017). Disseminated strongyloidiasis is highly fatal if left untreated, hence timely diagnosis and speedy management is highly crucial. Thus, in immunocompromised patients, the possibility of dissemination occurring should be considered and rapid diagnosis and treatment are essential to avoid fatality.

1.6 Predisposed Conditions Favouring Progression to Severe Strongyloidiasis

The transition from uncomplicated *Strongyloides* infection to severe strongyloidiasis may be aided by a number of factors that impair immune responses. The most common clinical conditions correlated with hyperinfection syndrome and disseminated are corticosteroids treatment, concomitant pathologies, and solid organ transplantation.

1.6.1 Corticosteroids Associated with Strongyloidiasis

The correlation between severe strongyloidiasis and corticosteroids has been well-documented in the scientific literature (Fardet et al., 2006). The pharmacologically induced conditions which include the treatment of autoimmune and inflammatory diseases and their therapy to avoid transplant rejection are most commonly associated with hyperinfection syndrome. Two- or three-fold increase in the risk of severe strongyloidiasis has been reported with corticosteroid therapy (Davidson et al., 1984). The long-term use of corticosteroids in the treatment of systemic lupus erythematosus (SLE) patients contributes to the establishment of lethal hyperinfection syndrome with systemic candidiasis (Byard et al., 1993). Furthermore, hyperinfection syndrome with mechanical ileus develops in patients having chronic obstructive pulmonary disease (COPD) treated with oral steroid therapy (Rothe et al. 2020). To date, there is no solid data on the mechanisms related to the conversion of uncomplicated to severe strongyloidiasis as a result of corticosteroid. However, the most likely explanation would be that lymphocyte activation and corticosteroids depress eosinophilia that are crucial for the control of *S. stercoralis* disease (Toledo et al., 2015). Corticosteroids may have a direct effect on *Strongyloides* parasites rather than the immune system, according to some researchers (Machado et al., 2011).

According to other theories, corticosteroids may act as larvae molting signal or revive reproductively latent females (Genta, 1992; Mansfield et al., 1996). Regardless of the amount, duration, or mode of administration, it is clear that corticosteroids cause hyperinfection syndrome (Krolewiecki & Nutman, 2019).

1.6.2 Concomitant Pathologies of Severe Strongyloidiasis

Conditions that have been linked with the increased risk for hyperinfection syndrome or disseminated strongyloidiasis include human immunodeficiency virus (HIV), human T- lymphotropic virus-1 (HTLV-1), chronic obstructive pulmonary disease, diabetes, malnutrition, systemic lupus erythematosus, and haematologic malignancies (Safdar et al., 2004; Marcos et al., 2011; Gonzalez-Ibarra et al., 2014; Yung et al., 2014; Walker et al., 2015; Rothe et al., 2020). Among these pathologies, the most associated risk factor for hyperinfection and disseminated strongyloidiasis is HTLV-1 infection (Carvalho et al., 2004; Gotuzzo et al., 2007).

Under usual occurrences, the reaction to parasitic infection is of the Th2 type, which is negatively regulated in HTLV-1. Strongyloides co-infection in HTLV-1, patients facilitate disseminated strongyloidiasis (Salles et al., 2013). It has been postulated that HTLV-1 infection induces Th1 bias in the immune system rather than the Th2 response that is essential for the removal of *S. stercoralis* infection (M Satoh et al., 2002). Other postulations also found that HTLV-1 is associated with changes in T-cell regulatory level, decrease in antigen-driven interleukin (IL-5) and parasite-specific IgE levels that have a protective role against *Strongyloides* infection (Hayashi et al., 1997; Porto et al., 2001; Montes et al., 2009). There also seems to be a bidirectional correlation between HTLV-1 infection and *S. stercoralis*, where *S. stercoralis* infection facilitates the HTLV-1 replication (Ratner et al., 2007). It is also

reported that HTLV-1 decreases the immunological control and is responsible for the severity of strongyloidiasis (Lowe et al., 2020). Patients with HTLV-1 acquire strongyloidiasis 2.4 times more often than non-infected patients, with a concurrent infection of 4 times higher in female than male (Tanaka et al., 2016).

Although strongyloidiasis was once associated with AIDS, there is no evidence of a link between HIV and strongyloidiasis (Siegel et al., 2012). HIV is linked with progressive CD4 lymphocytopenia that provide lower chance of *S. stercoralis* larvae to mature in the gut and decrease risk for autoinfection (Bar-Yoseph et al., 2017). The *Strongyloides* hyperinfection in HIV-infected individuals is mainly due to corticosteroid therapy (Keiser & Nutman, 2004). Urban HIV clinics in the United States reported 25% prevalence rate of strongyloidiasis among HIV patients (Nabha et al., 2012).

A significant positive correlation has been reported for diabetes with associated risk for strongyloidiasis (McGuire et al., 2019). There are several findings of disseminated strongyloidiasis in diabetes patients who have not been immunosuppressed, suggesting that the impaired immune system in diabetics is unable to control chronic strongyloidiasis (Lam et al., 2006; Murali et al., 2010; Rets et al., 2013).

1.6.3 Association of Strongyloidiasis with Solid Organ Transplantation

Strongyloidiasis in solid organ transplantation (SOT) is linked with a *de novo* acquisition, reinfection, reactivation of latent infections in recipients, or transmission of infections from donors. In the transplant population, *S. stercoralis* infection is associated with immunosuppression, both in solid organ and hematologic transplants (Krolewiecki & Nutman, 2019). Multiple cases have been reported in solid organ transplants since 1971 when the first case of strongyloidiasis occurred in a kidney transplant recipient (Fagundes et al., 1971). Besides the kidney, *Strongyloides* hyperinfection syndrome in transplant recipients have also been reported in liver (Vilela et al., 2009), heart (Schaeffer et al., 2004), pancreas (Ben-Youssef et al., 2005), intestine (Patel et al., 2008), lungs (Balagopal et al., 2009), and hematopoietic stem cell (Peixoto et al., 2019). A single donor was found to be the source of infection in three organ recipients which included the liver, pancreas and kidney (Rodriguez-Hernandez et al., 2009; Hamilton et al., 2011). The fatal consequences of donor derived strongyloidiasis have been found in pancreas, kidney, liver, heart (Mizuno et al., 2009; Hasan et al., 2013; Galiano et al., 2016). The onset of clinical manifestation of strongyloidiasis is usually reported at around six weeks up to nine months post-transplantation (Le et al., 2014). *Strongyloides* hyperinfection syndrome have also been reported in patients who were asymptomatically infected prior to engraftment (Snydman et al., 2009).

The New York Organ Donor Network recommends a new strategy to screen both organ donors and recipients for *Strongyloides* infection. It includes a template protocol to organ procurement centres to facilitate donor screening. Screening of potential donors with the implemented strategies discovered that 4.3% of the donors were positive for *S. stercoralis* (Abanyie et al., 2015). A majority of the recipients

from the *S. stercoralis*-exposed donors were treated for strongyloidiasis, consequently no recipient developed the disease. This shows that pre-transplant donor screening is an effective strategy to prevent donor-related strongyloidiasis (Abanyie et al., 2015). Meanwhile, solid organ recipients have also been recommended to screen for strongyloidiasis with targeted serological testing by the American Society of Transplantation, the Infectious Diseases Society of America, and the Centres for Disease Control and Prevention (Snydman et al., 2009; Schwartz et al., 2013; Levi et al., 2014). Current guidelines suggest a serological testing (or stool examination in selected cases) of both donor and recipient before transplantation (Snydman et al., 2009).

1.7 Host Immune Response against Strongyloidiasis

There is no detailed investigation of human immune responses to *S. stercoralis*. Most of what we know on immune response to this parasite comes from animal experiments. The immune system controls and eliminates the helminth infection by the host Th2 responses. The Th2 response is also crucial for the prevention of hyperinfection and disseminated strongyloidiasis in infected patients (Porto et al., 2001; Iriemenam et al., 2010). The Th2 response incorporates a range of cells such as neutrophils, eosinophils, epithelial cells, B cells, and molecules like mucins and cytokines. The response of the host immune system is characterized by two strategies, i.e., adaptive and innate immune responses (Breloer et al., 2017).

1.7.1 Innate Immune Responses to *Strongyloides* infection

Innate immunity is part of the immune system characterized by a non-specific and rapid response to the invading pathogen. The innate immune response in mice against *S. stercoralis* filariform larvae is portrayed in Figure 1.2. It is carried out by eosinophils, neutrophils, and macrophages that accumulate at the infection site (Hayes et al., 2018). The eosinophils and IL-5 play primary roles with neutrophils and macrophages playing accessory roles (Watanabe et al., 2000; Galioto et al., 2006). In *Strongyloides* infection, the eosinophil plays a dual function. The first function is the innate cytolytic activity against the eggs, larvae, and adult females (Mobley et al., 2017), while the second function serves as antigen presenting cells (APC) playing a crucial role at the crossroads of adaptive and innate immune responses. Interleukin (IL-5) serves an important role in the differentiation and maturation of eosinophils and its blockade impaired *S. ratti* worm clearance in the murine model (De'Broski et al., 2000). Eosinophils induces the production of protective antibodies in combination with the complement system, while other cells eliminate the infection (De'Broski et al., 2000). In mice the eosinophils kill *S. stercoralis* filariform larvae through the release of major basic protein (MBP) while in human the eosinophil cationic protein (ECP) are able to do this function (Rotman et al., 1996; O'Connell et al., 2011). Furthermore, there are two mechanisms for the eosinophil killing of *Strongyloides*: (i) direct killing mechanism that requires MBP and complement system, and (ii) indirect killing mechanism that requires interaction with other cells but does not require MBP (O'Connell et al., 2011).

Neutrophils are effector cells that kill the larvae through neutrophil-specific granular proteins called myeloperoxidase (Galioto et al., 2006). The mechanism of neutrophil activation is through CXCR2 receptors (O'Connell et al., 2011).

Neutrophils stimulation with soluble extract of *S. stercoralis* results in significant neutrophil recruitment. The recruited neutrophils, via chemokinesis and chemotaxis, induce the release of CXCR2 receptors ligands, phosphatidylinositol 3-kinase, and tyrosine kinase (Stein et al., 2009; O'Connell et al., 2011). In mouse, the killing of larvae by neutrophil through myeloperoxidase-dependent mechanism alone is sufficient if other cells are absent (O'Connell et al., 2011). However, in humans, the neutrophil is unable to kill *Strongyloides* larvae and can only reduce the motility of the parasite (De Messias et al., 1994).

The complement system is also needed for innate protective immunity against *S. stercoralis* in mice (Brigandi et al., 1996). The complement system can be activated by live *S. stercoralis* larvae via the classical and alternative pathways (De Messias et al., 1994). C3b, a component of the C3 complement system, causes neutrophils and monocytes to attach to the surface of *S. stercoralis* and facilitates degranulation and activation of cells that aid in larvae killing (Bonne-Année et al., 2011). The complement system aids in the binding of macrophages to the *S. stercoralis* larvae surface (De Messias et al., 1994). Macrophages, together with neutrophils and complement system, have been shown to eradicate *S. stercoralis in vitro* (Bonne-Année et al., 2013).

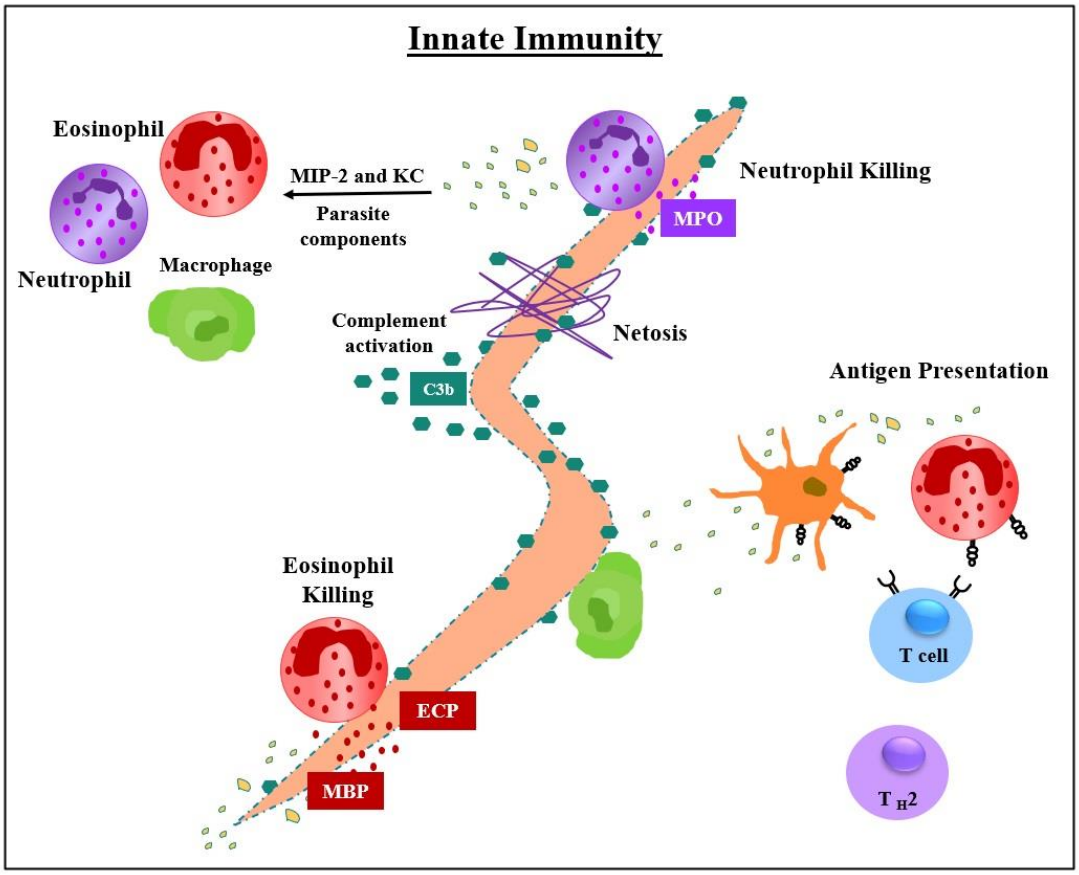

Figure 1.2 Innate Immunity to *S. stercoralis* filariform larvae in mice.

Parasite components that are necessary for innate immunity specifically engage eosinophils. Eosinophil granular proteins, major basic protein (MBP) in mouse, and eosinophil cationic protein (ECP) in human are toxic to *S. stercoralis* filariform larvae. Eosinophils also serve as antigen-presenting cells (APCs), causing Th2 cells to be activated. The parasite-specific recruitment of neutrophils to the L3 microenvironment leads to neutrophil production of additional chemokines such as keratinocyte chemoattractant (KC) and macrophage-inflammatory neutrophil (MIP-2). In an innate reaction, neutrophils activate myeloperoxidase to kill the larvae. Eosinophils and neutrophils kill larvae in a C3-dependent manner. C3b enhances effector cell adhesion to L3 (Breloer et al., 2017).

1.7.2 Adaptive Immune Responses to Strongyloidiasis

The responses of the adaptive immune system are highly specific and have lasting protection, characterized by cell mediated and antibody related responses. Figure 1.3 shows the adaptive immunity to *S. stercoralis* filariform larvae.

A parasite must meet the following criteria to trigger a cell-mediated adaptive immune response: (i) presented by APCs to T cells, (ii) dissociated with immunogenic peptides, or (iii) killed (Breloer et al., 2017). Eosinophils, which operate as antigen-APCs, stimulate the adaptive immune response by stimulating CD4+ T cells to generate the cytokines IL-4 and IL-5. Eosinophils also release IL-5, which stimulates B cell antibody production (Padigel et al., 2006; Weatherhead et al., 2014). Mice lacking the Th2-associated cytokine IL-4 or IL-5 have a harder time killing *S. stercoralis* larvae, indicating the relevance of CD4+ T cells in adaptive protective immunity to *S. stercoralis* larvae (Rotman et al., 1997). The method by which IL-4 and IL-5 contribute to adaptive response is, however, unknown. IL-5 is necessary for the generation of eosinophils in the innate response and IgM antibodies in the adaptive response (De'Broski et al., 2000). In the adaptive immune response, eosinophils are necessary for generating antibody formation and complement activation, but they do not kill parasites to accelerate the parasite killing (Breloer & Abraham 2017). Neutrophils uses toll-like receptor 4 (TLR4) and myeloperoxidase for killing, while macrophages are responsible for antibody-dependent killing. The overlapping effector role of eosinophils, neutrophils, and macrophages during the primary immune response is further precipitated by Th2 cell cytokines or through the creation of parasite-specific antibodies. All these effectors promote the elimination of *S. stercoralis* and prevent reinfection to ensure the host survival through a decrease in worm burden (Breloer & Abraham 2017).

The activation of B cells allows the production of parasite-specific IgM, IgA, IgE, IgG, and their subclasses (IgG1–IgG4), which are important for parasite killing. A rapid production of IgE, IgG1, IgG2, and IgG3 occurs in patients infected with *S. stercoralis*, followed by an increase in IgG4 level from weeks to months post-infection (Atkins et al., 1997; Atkins et al., 1999).

IgM antibodies to *Strongyloides* infection are observed in the first week of exposure, reach a peak in two to three weeks, then gradually subsides (Grove et al., 1982). Eosinophils induce IgM production with related cytokines IL-4 and IL-5. IgM production is inhibited in mice deficient with eosinophil, however, it is restored with a transfer of IL-4 expressing eosinophils (Wang et al., 2008). The main function of the parasite-specific IgM is control and eradicate migrating larvae in the tissue, in collaboration with macrophages (Breloer & Abraham, 2017). IgM can be recovered from mice one week after initial immunization by passively transferring protective immunity via a mechanism dependent on complement and granulocyte (Brigandi et al., 1996). IL-4 has a vital role in the class switching of IgM producing B cells to IgG and IgE (Shapira et al., 1991; Tangye et al., 2002).

IgA is the most frequent antibody class found in mucous membrane and secretions (Van Egmond et al., 2001; Pasala et al., 2015). In *S. stercoralis* infection, it includes lung and intestinal mucosa, therefore the host can produce local and systemic responses mediated by IgA (Costa et al., 2003). Deficiency of IgA facilitates *Strongyloides* infection in humans and animals, and mice immunised with live *S. stercoralis* live larvae have higher IgA levels (Mansfield and Schad 1992; Abraham et al. 1995; Mansfield et al., 1996). Strongyloidiasis patients' breast milk and saliva contain the IgA antibodies, and can also be found in their serum (Ribeiro et al., 2010).