PROTEOMIC AND TRANSCRIPTOMIC ANALYSES OF PROTEIN BIOMARKERS DURING EARLY HYPERINFECTION IN STRONGYLOIDIASIS

KHALID JAMEEL KADHIM AL-ZIHIRY

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By

KHALID JAMEEL KADHIM AL-ZIHIRY

Thesis Submitted to the School of Graduated Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

January 2016

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DEDICATIONS

My Parents

My wife

My Supervisor

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Doctor of Philosophy

PROTEOMIC AND TRANSCRIPTOMIC ANALYSES OF PROTEIN BIOMARKERS DURING EARLY HYPERINFECTION IN STRONGYLOIDIASIS

By

KHALID JAMEEL KADHIM AL-ZIHIRY

January 2016

Chair : Ngah Zasmy Unyah, PhD
Faculty : Medicine and Health Sciences

Strongyloides stercoralis is the most common intestinal nematode in humans, and it infects millions of people worldwide. This infection results in asymptomatic chronic disease that may remain undetectable. Its unique ability to proliferate within the host can cause a hyperinfection syndrome and dissemination of infective larvae in individuals with impaired cell-mediated immunity, thus increasing the mortality rate up to 87%. The diagnosis of hyperinfection syndrome is difficult to establish and entails a high level of suspicion. The main objective of the current study was to identify specific protein biomarkers from the excretory/secretory (ES) products of the infective filariform larva and from the serum proteins of infected Wistar rats that can be used as diagnostic indicators for early hyperinfection syndrome in strongyloidiasis. In this study, *S. ratti*, which is an animal parasite similar to *S. stercoralis* and commonly used in research related to *S. stercoralis*, was used as a model. Seventy wild rats, *Rattus norvegicus*, were trapped from different locations in the Serdang areas and were brought back to the Animal Experimental Unit and Medical Parasitology and Entomology Lab in UPM for further examination. *S. ratti* was detected in 34.2% of the trapped wild rats by using different conventional parasitological techniques and this was then reconfirmed by using Polymerase chain reaction (PCR) methods. A PCR method targeting the rRNA gene of the species of *Strongyloides* was conducted based on the published universal primers for the detection of *S. ratti* in faecal samples of wild rats. *Strongyloides ratti* was then isolated from the wild rats and new colonies were established and maintained in laboratory bred Wistar rats for the continuous supply of *S. ratti*. Faecal pellets from infected Wistar rats were collected and cultured using Modified Faecal Filtration Culture technique (MFFC) for the harvesting of high quality and quantity of infective filariform larvae (L3) used for this research. An experimental study was performed to induce hyperinfection syndrome and dissemination of L3 larvae of *S. ratti* in experimentally immunosuppressed Wistar rats using prednisolone, a corticosteroid immunosuppressive drug to validate that the pathological changes that occurred were similar between the Wistar rat and human strongyloidiasis. Infected Wistar rats were sacrificed and tissue samples were collected for histopathology study. Prednisolone treatment resulted in a dramatic increase in the infection intensities as proved by the increased in eggs and larval output, and adult recovery that exceeded the inoculated doses. The results of the histopathology study showed dissemination of infective filariform larvae mainly in the tissues of the lungs and liver because of an increased parasite burden during

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hyperinfection and disseminated strongyloidiasis. These observations were similar to human strongyloidiasis under immunosuppressive or anti-inflammatory regimens, including corticosteroid therapies. In this study, the Wistar rats were divided into two main groups, the first group was infected with *S. ratti* but without giving prednisolone (non-treated group) and the second group was infected with *S. ratti* and treated with prednisolone (treated group). Excretory/secretory (ES) products from the filariform larvae and blood serum from the non-treated Wistar rats and the Wistar rats treated with 4.5 mg/kg prednisolone were analysed in one-dimensional and two-dimensional gel electrophoresis (1D, 2D), LC-MS/MS and MALDI-TOF/TOF MS. A total of 10 protein biomarkers were detected as overexpressed from a treated ES product with molecular weights ranging from 30-90 kDa and isoelectric points ranging from 3-11, as well as 8 overexpressed protein biomarkers which were detected from treated Wistar rat sera. Relative semi-quantitative real-time PCR (qPCR) with SYBR Green was performed using a Mastercycler Realplex to compare the expression level of the detected biokmaker's genes between treated and non-treated groups. These genes were selected based on proteomics results and are representing respective protein that have identified earlier, in order to validate the related expressed protein biomarkers. Nine related genes were identified which showed significantly higher expression levels to the related identified biomarkers, whereas one gene (*Arg*) recorded down regulation in its expression. The study concluded that, 18 protein biomarkers were successfully identified and could be used as diagnostic biomarkers during early hyperinfection syndrome in strongyloidiasis.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

ANALISIS PROTEOMIK DAN TRANSKRIPTOMIK TERHADAP BIOMARKER PROTEIN SEMASA PERINGKAT AWAL JANGKITAN-HIPER DALAM STRONGILOIDIASIS

Oleh

KHALID JAMEEL KADHIM AL-ZIHIRY

Januari 2016

Pengerusi : Ngah Zasmy Unyah, PhD
Fakulti : Perubatan dan Sains Kesihatan

Strongyloides stercoralis adalah merupakan nematod usus yang biasa terdapat dalam manusia, dan ia menjangkiti berjuta-juta manusia di seluruh dunia. Jangkitan ini menghasilkan penyakit kronik yang asimptomatik serta sukar untuk dikesan. Keupayaan unik parasit ini untuk berkembang dengan sendirinya di dalam perumah boleh menyebabkan sindrom jangkitan-hiper dan jangkitan-penyebaran oleh larva infektif pada individu yang keimuniti sel-perantara terjejas, justeru itu akan meningkatkan kadar kematian sehingga 87%. Diagnosis terhadap jangkitan-hiper sukar dilakukan dan boleh menyebabkan diagnosis yang kurang tepat. Objektif utama kajian ini adalah untuk mengenal pasti biomarker protein yang spesifik dari produk rembesan/perkumuhan (ES) dari larva infektif dan serum tikus Wistar yang boleh digunakan sebagai petanda diagnostik untuk mengesan peringkat awal jangkitan-hiper dalam strongiloidiasis. Dalam kajian ini, *S. ratti* yang merupakan parasit dalam haiwan dan hampir sama dengan *S. stercoralis* serta sering digunakan di dalam kajian *S. stercoralis* telah digunakan sebagai model. Tujuh puluh ekor tikus liar, *Rattus norvegicus*, telah berjaya diperangkap dari lokasi berbeza di sekitar Serdang dan dibawa ke Unit Ujikaji Haiwan dan Makmal Parasitologi dan Entomologi Perubatan untuk kajian selanjutnya. Sejumlah 34.2% *S. ratti* telah dikesan daripada jumlah keseluruhan tikus liar yang telah diperangkap menggunakan teknik parasitologi konvensional yang berbeza dan telah disahkan dengan menggunakan Reaksi rantai polimerase (PCR). Satu kaedah PCR mensasarkan subunit kecil gen rRNA bagi sepsis *Strongyloides* telah dioptimumkan dengan menggunakan primer yang telah diterbitkan untuk mengesan *S. ratti* di dalam tinja tikus liar. *Strongyloides ratti* kemudiannya diasingkan dari tikus liar dan koloni baru telah diwujudkan dan diternak di dalam tikus Wistar untuk mendapatkan bekalan berterusan larva infektif *S. ratti*. Pelet tinja dari tikus Wistar yang telah dijangkiti akan dikutip dan dikultur dengan menggunakan teknik Kultur Penapisan Tinja Terubah (MFFC) untuk bekalan berkualiti dan berkuantiti tinggi larva infektif filarifom yang digunakan dalam kajian ini. Satu kajian telah dilakukan untuk merangsang jangkitan-hiper dan jangkitan-penyebaran oleh larva L3 *S. ratti* di dalam tikus Wistar imun-tertindas melalui penggunaan prednisolone, yang merupakan sejenis ubat perangsang imun-tertindas kortikosteroid untuk mengesahkan perubahan patologi yang serupa memang berlaku diantara tikus Wistar dan strongiliodiasis pada manusia. Tikus Wistar yang dijangkiti telah dikorbankan dan sampel-sampel tisu telah diambil untuk kajian histopatologi. Rawatan Prednisolone menyebabkan peningkatan jangkitan yang dramatik

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melalui pembuktian peningkatan bilangan telur dan penghasilan larva, dan pembentukan cacing dewasa yang didapati melebihi dos-dos disuntik asal. Keputusan kajian histopatologi menunjukkan penyebaran larva infektif filariform lebih tertumpu dalam tisu paru-paru dan hati akibat beban parasit yang meningkat semasa jangkitan-hiper dan jangkitan-penyebaran dalam strongiloidiasi. Pemerhatian ini adalah sama seperti yang berlaku di dalam manusia akibat strongiloidiasis akibat penggunaan ubat perangsang imun-tertindas, termasuk terapi kortikosteroid. Dalam kajian ini, tikus Wistar telah dibahagikan kepada dua kumpulan utama, kumpulan pertama adalah kumpulan yang telah dijangkiti dengan *S. ratti* tetapi tanpa Prednisolone (kumpulan tanpa-rawatan) dan kumpulan kedua juga dijangkiti dengan *S. ratti* termasuk pemberian Prednisolone (kumpulan rawatan). Produk rembesan/ perkumuhan (ES) dan serum darah dari tikus tanpa-dirawat dan tikus telah dirawat dengan 4.5mg/kg Prednisolone dianalisis menggunakan elektroforesis gel dua-dimensi (1D, 2D), LC-MS/MS dan MALDI-TOF/TOF MS. Sejumlah 10 biomarker protein yang dirembeskan secara berlebihan telah dikenal pasti daripada produk ES yang mempunyai berat molekul antara 30-90kDa dan titik isoelektrik di antara 3-11, manakala 8 biomarker protein yang dirembeskan secara berlebihan telah dikenal pasti dari serum tikus Wistar yang telah dirawat. Relatif-kuantitatif masa nyata PCR (qPCR) dengan SYBR hijau telah dilakukan menggunakan alat Mastercycler Realplex (Eppendorf) untuk membandingkan tahap ekspresi protein biomarker yang telah dikenal pasti daripada gen biomarker di antara sampel yang dirawat dan tanpa-rawat. Sembilan gen telah dikenal pasti menunjukkan tahap ekspresi yang tertinggi, manakala satu gen (Arg) mencatatkan nilai ekspresi yang terendah. Kesimpulan hasil kajian menunjukan, 18 biormarker protein telah berjaya dikenal pasti dan mungkin dapat digunakan sebagai petanda diagnostik semasa mengesan jangkitan-hiper pada peringkat awal dalam strongiloidiasis.

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.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Ngah Zasmy Unyah, Ph.D
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Roslaini Abd Majid, Ph.D
Senior Lecturer
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Rukman Awang Hamat, MD, MPath
Associate Professor
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

Signature: _____
Name of Chairman of Supervisory Committee: Ngah Zasmy Unyah, Ph.D

Signature: _____
Name of Member of Supervisory Committee: Roslaini Abd Majid, Ph.D

Signature: _____
Name of Member of Supervisory Committee: Rukman Awang Hamat, MD, Path

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APS	Ammonium persulfate
A_{260}/A_{280}	Absorbance reading at OD_{260} and OD_{280}
BLAST	Basic Linear Alignment Search Tool
bp	Base pair
°C	Degree centigrade
cDNA	Complement Deoxyibonucleic acid
CHAPS	(3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)
Ct value	Threshold line generated by real-time PCR
ddH_2O	Double distilled water
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Diothiothreiol
EDTA	Ethylenediaminetetraacetic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	hour
HSP	Heat shock protein
H_2O	Water
IEF	Isoelectric focusing
IPG	Immobilised pH gradient
kDa	Kilo Dalton
LC/MS	Liquid chromatography–mass spectrometry
MALDI	Matrix-assisted laser desorption/ ionisation

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min	Minute
mg	Milligram
mM	Millimolar
mg/L	Milligram/litre
ml	Milliletre
mRNA	Messenger RNA
µg	Microgram
lµ	Microlitre
mµ	Micrometer
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nm	Nanometer
OD	Optical density
PBS	Phosphate buffered Saline
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
pI	Isoelectric point
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
RT-qPCR	Reverse transcription quantitative PCR
R^2	Correlation coefficient
S	Second (time)
SDS	Sodium dodecyl sulphate
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel

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	electrophoresis
Spp.	Species
TBE	Tris-borate EDTA
TOF	Time of flight
Tris	tris(hydroxymethyl)amino- methane
UV	Ultra Violet
V	Voltage
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume
X	Times
α	Alpha
β	Beta

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CHAPTER 1

GENERAL INTRODUCTION

Nematodes are successful, widely distributed parasites, many of which evolved a parasitic life cycle as pathogens of human and animals. Hundred millions of persons are currently infected in the world (Kramme *et al.*, 2011). The vast majority among the poorest world countries and 3.5 million are exposed to these parasites mostly due to gastrointestinal nematodes (Hotez, 2008).

The genus *Strongyloides* consist of more than 40 species of helminth parasites of human, birds, amphibians and reptile hosts, whereas the majority are parasites of domesticated mammals, include dogs, cats and livestock (Dorris *et al.*, 2002). Human infections with the nematode *S. stercoralis* are frequent in poor hygienic areas and in humid and warm environment. It is widespread in tropical and subtropical areas. However, it may present in temperate countries with favourable conditions. *S. stercoralis* infection can be also identified in non-endemic countries due to the migration and travel. The infection is more common in migrants than in travellers (Bethony *et al.*, 2006). *Strongyloides stercoralis* infection belongs to the heterogeneous group of under-researched neglected tropical diseases (NTDs) (Feasey *et al.*, 2010; Vanderelst & Speybroeck, 2010) with no available burden estimation (Utzinger *et al.*, 2009) mainly due to the lack of accurate diagnostic tools which obviously explains the often unsatisfactory epidemiological mapping of strongyloidiasis (Bergquist *et al.*, 2009; Johansen *et al.*, 2010).

An estimatimation of 30-100 million infection with *S. stercoralis* worldwide. However, little information is available about emerging consequences in endemic areas and the epidemiological data are scarce in tropical countries. The higher suspicion index of *S. stercoralis* infection is potentially improves the early diagnosis and effective treatment (Becker *et al.*, 2011; Knopp *et al.*, 2009).

Life cycle

Strongyloides stercoralis has a complicated life cycle, adult parasitic females are the parasitic phase which live embedded inside the small intestine mucosa. The eggs produced and released by these parasitic females are passed out of the host body with the excreted stools. In the external environment, the eggs can be developed in two ways: homogonic (direct or parasitic) and heterogonic (indirect life cycles or free-living generation). In homogonic cycle, rhabditiform larvae moult twice into infective filariform larvae (iL3s), which able to infect a new host through skin penetration. The filariform larvae are developmentally arrested stage and no further development occurs until they encounter a new host. In heterogonic cycle on the other hand, rhabditiform larvae (L2) of the second-stage moult through four larval stages and develop into free-living L3 and L4 stages to mature as free-living adult females and males. Free-living females and males mate and reproduce sexually in the environment to produce eggs which will develop into the infective L3 as occurs in the homogonic cycle. These larvae from both cycles may infect a new host, and

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during migration through the host's body, they moult twice to L4 stage and adult parasitic females dwell in the gut. They start producing eggs, which pass to stool, and the direct and indirect cycle will repeat again (Santos & Merlini, 2010; Viney, 1999) (Figure 1.1).

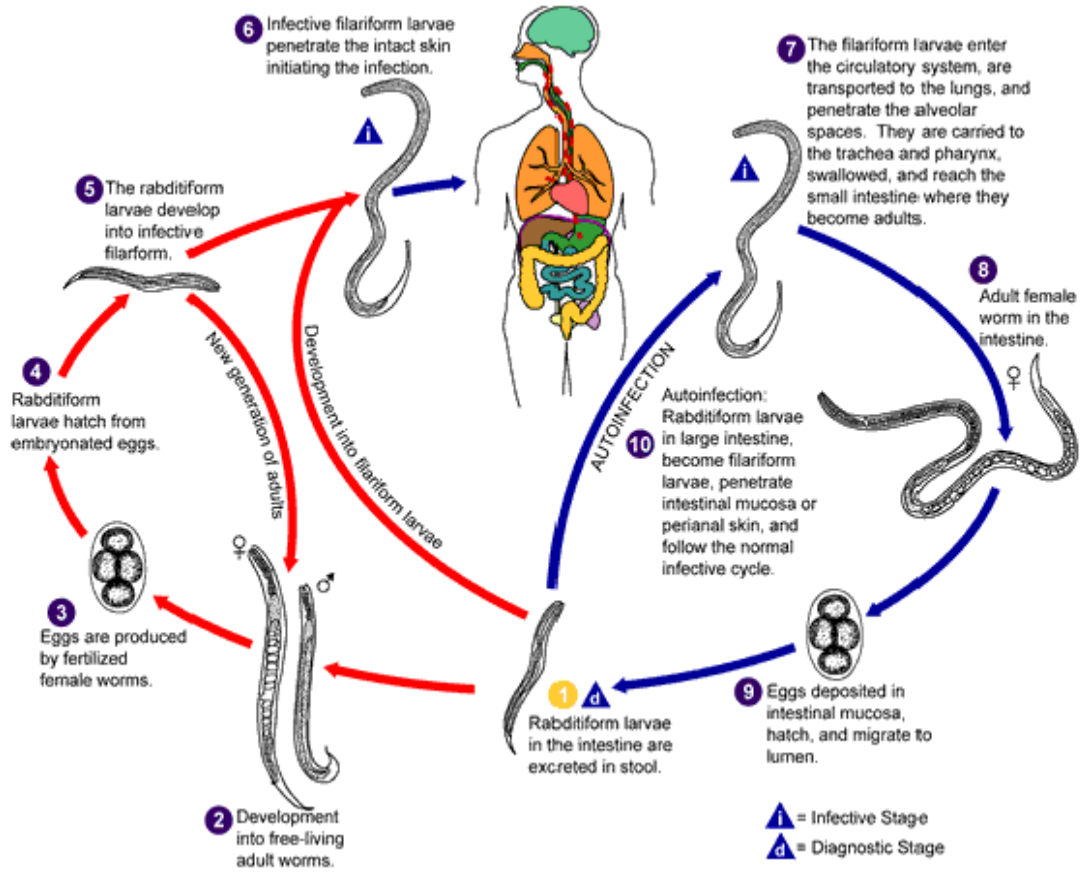

Figure 1.1: *Strongyloides stercoralis* life cycle (CDC, http://www.cdc.gov).

Symptoms

The most common signs of symptomatic strongyloidiasis include lower abdominal pain, vomiting, intermittent or persistent diarrhoea, fever, indigestion, rash, anal pruritus, malabsorption, cramping constipation, weight loss, gastrointestinal ulceration, perforation, or obstruction, and haematemesis. Some patients may suffer from asthma-like cough and wheezing. Peripheral eosinophilia may often be the first sign of this parasitic infection (Koczka *et al.*, 2012). A recent information through questionnaires and evaluations of stool samples indicated that the persons infected with strongyloidiasis were more likely to complain of stomach aches (Becker *et al.*, 2011). Based on the evaluations of the gastrointestinal tract pathogenesis, there is a lack of lesions in the mucosa in most infections, which usually becomes apparent in severe cases. These changes represent an inflammatory response which includes congestive catarrhal enteritis to edematous and ulcerative enteritis. A bothersome symptom and nonspecific urticaria were a very frequent finding (Krolewiecki *et al.*, 2013).

Immunity

Parasitic infections represent a competition between the parasites and their hosts. The important difference between parasites of vertebrates and free-living organisms is that the parasites dwell inside their hosts and therefore must survive in the face of a complicated immnity directed against them (Wakelin, 1996). The most important test for the parasites is how to invade their host challenges and to successfully migrate to their final destination. This process usually requires passing through host tissues, basement membranes, extracellular matrices, and blood or lymph vessel walls. Parasites often produce an array of substances that can interfere with the host´s immune system (Nagaraj *et al.*, 2008). The ability of helminths to modulate the defence system supports their longevity in their host (Yazdanbakhsh *et al.*, 2002). This modulation mechanism is most likely caused by releasing of soluble mediator molecules which degrade, ligate or interact with certain cells of host immune system (Hewitson *et al.*, 2009). The main expressed and secreted substances from nematodes are proteases, protease inhibitors, antioxidants and orthologs of host cytokines and their receptor molecules which modulate host immune responses (Bungiro & Cappello, 2005; Younis, 2011).

During innate and adaptive immune response, eosinophils and antibodies play important role in the immune system against *S. stercoralis* larvae (Mir *et al.*, 2006; Satoh *et al.*, 2004). *S. stercoralis* antigens activate eosinophils; induce the expression of major histocompatibility complex (MHC II) and T-cell co stimulatory molecules. T cells can be stimulated by these eosinophils for antigen-specific immune response. Eosinophils are also believed to play a role as antigen presenting cells (APCs) for the induction of the primary and secondary T-helper 2 (Th2) immune responses to *S. stercoralis* (Padigel *et al.*, 2006) which represents a link between innate and adaptive immune responses. Both neutrophils and eosinophils were reported to have an essential role in protective innate immunity. However, only neutrophils are essential to produce a protective adaptive immune response against the larvae of *S. stercoralis* (Galioto *et al.*, 2006). In severe strongyloidiasis individuals, eosinophils levels were reported to be lower than that of asymptomatic individuals. Thus, eosinophils may play a key role in preventing *S. stercoralis* infection (Iriemenam *et al.*, 2010).

Acquired immunity has been found to develop in patients against *S. stercoralis* infection, related to the development of antibody responses to the infection (Iriemenam *et al.*, 2010). Furthermore, this acquired protective immunity against the infection has been widely described in laboratory animal models (Bonne-Année *et al.*, 2011). However, the nematode longevity in humans argues that the parasite burden could be limited by the host immune response, but it is not a sufficient response to eliminate parasitic infection.

Experimental studies have demonstrated that eosinophils have an essential role in protective mechanism against *S. stercoralis* along with the cellular and humoral immunity, but the mechanism regulating eosinophils are still not known (Mir *et al.*, 2006).

The immune response against *S. stercoralis* is necessary for control of infection and the prevention of hyperinfection (Iriemenam *et al.*, 2010). High level of protective antibody dependent immunity was the result of immunisation of experimental mice

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with live larvae of *S. stercoralis* (Kerepesi *et al.*, 2004). Furthermore, immunised mice with soluble proteins extracted from the *S. stercoralis* larval stage also produced antibody-dependent protective immune response. Using antibodies from these protected mice, protective *S. stercoralis* antigens were isolated by affinity purification. These antigens, were then pooled and used to induce a significant protective immunity, with 83% reduction of the challenged larvae. Antibodies from humans with *S. stercoralis* chronic infection were also capable of killing *S. stercoralis* larvae.

Mice immunised with recognised antigens by a protective human IgG showed a 76% reduction in larval survival. The IgG pool was then used to test specific vaccine candidates. Three of which (Ss-EAT-6, and Ss-LEC-5 and SsTMY-1) could be characterised at a molecular level. One of them (Ss-EAT-6) induced a 35% larval survival reduction when tested in DNA-based immunisation protocols. Serum samples from immunised mice with the DNA encoding Ss-EAT-6 was effective to transfer this partial immunity (Kerepesi *et al.*, 2005).

Hyperinfection and Dissemination

Strongyloidiasis autoinfection is an essential characteristic feature of *S. stercoralis* life cycle. Instead of shedding in the human faeces, the first stage rhabditiform larvae (L1) molt twice inside the host and become filariform larvae which can penetrate the human intestine or perianal skin and disseminate to different organs of the host body, causing hyperinfection syndrome, dissemination infection which involves other organs (Vadlamudi *et al.*, 2006).

Human T cell lymphotropic virus 1 (HTLV-1) represents an important risk factor for the accelerating of hyperinfection syndrome or disseminated strongyloidiasis. High rates of strongyloidiasis hyperinfection were documented in HTLV-1 patients (Mejia & Nutman, 2012). Those Patients are especially susceptible to hyperinfection and disseminated cases and are likely to experience failure of standard therapy (Roxby *et al.*, 2009). The long-lasting vitamin B deficiency was also proposed to be the predisposing factor for hyperinfection in some cases (Marathe & Date, 2008).

Surprisingly, hyperinfection syndrome is not common in HIV positive patients (Keiser & Nutman, 2004). One of the hypotheses suggesting that HIV- associated immunodeficiency suppresses larval form development in the gut, preventing autoinfection (Viney *et al.*, 2004). Nevertheless, HIV infected patients remain at risk of hyperinfection, as they could receive corticosteroids for a variety of conditions such as *Pneumocystis jiroveci* pneumonia, extrapulmonary tuberculosis, and thrombocytopenia and furthermore, HIV patients can be coinfected with HTLV type I which increases the risk of having the hyperinfection syndrome (Karp & Auwaerter, 2007).

Massive infections with *S. stercoralis* may cause life-threatening risks with intestinal obstruction, meningitis, pneumonia and septicaemia. The mortality rate of disseminated strongyloidiasis has been recorded to be as high as 87% and 50% mortality despite therapy, with poor outcome in patients with hyperinfection (Al Maslamani *et al.*, 2009). Disseminated strongyloidiasis is usually associated with

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opportunistic bacterial infections. Penetration of tissues by large numbers of larvae often associated with microbial sepsis as these larvae carry organisms during their transmural migration across the intestinal wall reaching to the bloodstream (Mejia & Nutman, 2012). Bacterial infections of gastrointestinal *S. stercoralis* are rarely reported in the absence of dissemination (Al-Hasan *et al.*, 2007). Some microorganisms were reported to be the causative agents of sepsis in these patients, which include group Streptococci, Candida; *Streptococcus bovis, Klebsiella pneumonia, Escherichia coli, Pseudomonas* sp., *Proteus mirabilis, Enterococcus faecalis, S. pneumonia and coagulase-negative staphylococci.* The risk of microbial sepsis is increased when the patient is under immunosuppressive therapy (Mejia & Nutman, 2012).

Diagnosis

Diagnosis of *S.stercoralis* relies on detection and identification of the eggs and rhabditiform larvae in the stool using number of conventional techniques including faecal direct smear stained with Lugol iodine, Harada-Mori filter paper culture, Baermann concentration, and agar plate cultures. However, in most light cases of strongyloidiasis, the intestinal parasite load is very low and the emerging of the eggs and rhabditiform larvae is minimal; therefore, many uncomplicated and chronic infections are missed. The majority of the difficulties posed in the evaluation of infections and consequences *S. stercoralis* result from the challenging aspects of the diagnosis. Kato Katz technique and McMaster slide are both methods to quantitate eggs in the faeces, but could not detect rhabditiform larvae. A new development FLOTAC, an improvement of the McMaster technique, focused only on egg detection and failed to identify the presence of rhabditiform larvae in stool samples (Glinz *et al.*, 2010). The most commonly used diagnostic techniques are currently the Harada Mori methods and Baermann funnels. However, their sensitivity is not optimal. The agar plate culture (APC) method is more accurate, but also more expensive, time consuming and laborious (Siddiqui & Berk, 2001).

Low burden of larval stages in faecal samples in chronic strongyloidiasis negatively affects the performance of standard techniques. In more than 65% of cases, there were ≤25 rhabditiform larvae per gram (LPG) of faecal samples. It has been demonstrated that a single stool examination insufficient to identify larvae in approximately two-third of the cases studied. Repeating of stool specimen examinations increases the probability of parasite detecting. Diagnostic sensitivity can approach 50% and 100% rising if 3 and 7 serial stool samples are examined respectively (Montes *et al.*, 2010; Nielsen & Mojon, 1987; Pelletier 1984).

The count peripheral blood eosinophils are not an accurate indicator of parasitic infection. Eosinophilia is a common finding in the chronic stage of *S. stercoralis* patients, but in the immunocompromised individuals (patients receiving immunosuppressant therapy or with hyperinfection syndrome), the eosinophils count are unreliable (Al-Hasan *et al.*, 2007); because they usually show a suppressed eosinophils count (Keiser & Nutman, 2004), Nevertheless; individuals who have a high level of peripheral eosinophilia during hyperinfection seem to have a better prognosis (Marathe & Date, 2008).

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Serological diagnostic assays can simplify and overcome the poor sensitivity of *S. stercoralis* detection when single stool sample is examined. These assays performed for diagnosis of individual infections as well as for defining the disease prevalence at the community level (Ramanathan & Nutman, 2008). The enzyme-linked immunosorbent assay (ELISA) uses crude somatic antigen extract of *Strongyloides* to measures IgG responses in the patient's serum. The disadvantage of this technique is the time required to mount an immune response (4-6 weeks) and can also show false negative results in acute infections (Krolewiecki *et al.*, 2010). The test may continue to be positive for extended periods of time after the case treatment. Furthermore, cross-reactivity was reported in patients with active filarial infections (Ramanathan & Nutman, 2008).

Recombinant antigen-based assays introduced as an attractive alternative to the use of *Strongyloides* crude antigen which requires the maintenance of the nematode in the laboratory animals such as dogs and gerbils with chronic infections or stool collections from infected persons for antigen preparation. This recent technique can produce specific antigens in large quantities. Evaluation of NIE recombinant antigen (31kDa) revealed high specifity of strongyloidiasis detection and no cross-reactivity with other Soil-transmitted helminths (STH) was reported. The technique demonstrated an improvement in the test sensitivity compared to stool evaluations based on a single stool examination (Krolewiecki *et al.*, 2010).

Treatment

Drugs include albendazole, mebendazole, pyrantel/oxantel levamisole, and benzimidazoles are recommended for use in large scale interventions to treat and control STH infections. However, they are not effective against *S. stercoralis* infection, at least in single doses as recommended for preventive chemotherapy interventions (Albonico *et al.*, 2008). The unique life cycle of *S. stercoralis* due to the ability of the worm to reproduce inside a human host, leads to treatment failure with these common drugs (Krolewiecki *et al.*, 2013). Drug such as albendazole with approximately 40% of cure rates with single-dose regimens for STH is unsatisfactory option for strongyloidiasis (Keiser & Utzinger, 2008).

Ivermectin, the standard drug for strongyloidiasis treatment, showed superior efficacy in comparison to thiabendazole (Bisoffi *et al.*, 2011). This drug has a well determined safety profile during widespread use in lymphatic filariasis and onchocerciasis control programs . It is however limited in paediatric populations, restricting its use according to different criteria of weight, age, and/or height. The reason behind this is related to the potential toxicity of the central nervous system seen in some patients. Ivermectin also is not recommended in pregnancy period and the first week postpartum. Emerging data suggest that this drug could be a component of a combination regimen with albendazole or mebendazole in the treatment of *T. trichiura* (Knopp *et al.*, 2010). Despite the widespread use of ivermectin in many countries endemic for onchocerciasis, lymphatic filariasis and other STH, very few studies have focused on the impact of those interventions on *S. stercoralis* in co-endemic infection areas (Knopp *et al.*, 2009).

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The response to anti-helminthic drugs depends on a competent immune system and no response has been reported using multiple courses of commonly used drugs (Thiabendazole and ivermectin) in immunosuppressed hosts (Amornvipas *et al.*, 2010).

The difficulty of strongyloidiasis treatment is that the truly effective drug must completely eradicate this disease by killing every autoinfective L3 larvae which only removes the threat of potentially serious disease. These larvae are often resistant to most chemical agents in contrary to the common helminth infections. The treatment is considered sufficient if a nematode burden is below the level at which clinical disease develops. In *S. stercoralis*, however, the poor sensitivity of the diagnostic stool examination makes it more difficult to detect the treatment efficacy, because the positive findings of a follow-up stool examination must be the basis of the successful cure (Siddiqui & Berk, 2001).

Control

Parasitic diseases can be widely controlled in the developed countries by effective public sanitation and primary health care programs. However, in these countries, some parasitic diseases including human strongyloidiasis are still widespread and chemotherapy treatment does not protect and control the rapid reinfection (Tazir, 2009). The basis for the control of strongyloidiasis is the information available on the impact of this disease on the quality of life impaired in endemic areas instead of only in travellers (Becker *et al.*, 2011). The distribution map of strongyloidiasis in population based on the epidemiologic studies showed similar distribution as hookworms in adolescents, remaining stable in adults. Some studies have demonstrated that there is no gender difference. However, others have demonstrated it more prevalent in males, possibly due to differential exposure (Krolewiecki *et al.*, 2011).

Strongyloides ratti

Strongyloides stercoralis can successfully be maintained in gerbils (Nolan *et al.*, 1999) and dogs (Lok, 2007), but does not develop beyond the filariform larvae in mice (Hartmann *et al.*, 2012). The use of gerbils and dogs is costly and represents an ethical concern. The rat nematode *S. ratti* is an ideal parasite to work with in the laboratory in comparison to *S. stercoralis*. The experimental infections with this nematode have been used to model the complete life cycle in mice. *Strongyloides ratti*, has direct and indirect life cycle, and in indirect phase there is no insect vector required to complete the cycle. The absence of an insect vector allows study the nematode genes and their protein products by comparing the life cycle stages at the molecular level which is necessary in the processing the nematode infection or in its survival within the host (Grove, 1989) .

Strongyloides stercoralis does not mature to the adult stage in small laboratory animals such as the Wistar rat and therefore several researchers suggested that *S. ratti*, a natural parasite of rats can be used as a model for human strongyloidiasis in most experimental investigations due to its similarity in morphology and life cycle

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(Abadie, 1963) and antigenic composition (Li *et al.*, 2006) with *S. stercoralis*. However, it was uncertain as to whether hyperinfective or disseminated strongyloidiasis occur in murine infection of *S. ratti*. Furthermore, there was no indication of naturally occurring hyperinfection or dissemination in the published studies, and all the reported cases only deal with human strongyloidiasis.

The presence of both direct and indirect developments in *S. ratti* life cycle provides a powerful tool to explore the genetic variations associated with the evolution of helminth parasitism (Mello *et al.*, 2009).

Aim of study

Alteration the fatal course of *S. stercoralis* infection can be achieved by early detection of hyperinfection strongyloidiasis (Agrawal *et al.*, 2009); thus, there is rising need to develop sensitive and specific diagnostic test for early diagnosis of this stage of *S. stercoralis* infection even in patients with no clinical symptoms of immunosuppression, to avoid long suffering and the peril of serious, life-threatening hyperinfection.

Objectives

General objective

To identify biomarkers associated with the early hyperinfection syndrome of strongyloidiasis in animal models.

Specific objectives

1: To isolate and identify *S. ratti* from wild rat using conventional and molecular methods.

2: To induce the hyperinfection syndrome of strongyloidiasis in animal models.

3: To identify specific protein biomarkers secreted by the filariform larva during hyperinfection syndrome in 1D and 2D gel electrophoresis.

4: To analyse and validate the gene expressions of the identified biomarkers at transcriptomic level using quantitative real time PCR.

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LIST OF PUPLICATIONS

Khalid Jameel Kadhim Al-Zihiry ,Mahmuda Aliyu, Salman Sahab Atshan, Zasmy Unyah Zaid Osama Ibraheem, ,Roslaini Abdul Majid, Rukman Awang Hamat, Wan Omar Abdullah. (2015). Molecular detection of *Strongyloides ratti* in faecal samples from wild rats in Serdang area, Malaysia. *Trop. J. Pharm. Res.*, 14(7): 1167-1173.

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