UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND IMMUNODIAGNOSTIC APPLICATION OF 30 kDa MONOCLONAL ANTIBODY AGAINST COPROANTIGENS OF *Strongyloides* IN ANIMAL MODEL

NOOR ABDULHALEAM

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By

NOOR ABDULHALEAM

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

November 2017

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DEDICATION

This research work is dedicated with all humility and respect to:
The first human teacher Mohammed peace be upon him
Spirit of my late Mother and Sister
My father
My brothers and sisters
My husband and kids
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

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By

NOOR ABDULHALEAM

November 2017

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Faculty : **Medicine and Health Sciences**

Intestinal strongyloidiasis is usually underdiagnosed and the available parasitological techniques such as stool examinations based on Baermann or agar plate cultures are time-consuming, which involved three specimens collected at the different time intervals for more accurate and sensitive application. The current available preferred serological technique for diagnostic of intestinal strongyloidiasis shows the cross-reaction with other intestinal helminths. This preliminary study is aimed to produce and evaluate monoclonal antibody against the coproantigen of *Strongyloides ratti* in the animal model. *Strongyloides ratti* infection model was established and maintained in immunosuppressed Sprague Dawley rats. The saline extract protein from the infective larvae (iL3) was used as antigens for the immunisation of BALB/c mice. The splenocytes harvested from the immunised mice were fused with myeloma (SP2/0) cells for hybridoma production. Supernatants from the positive hybrids were screened by indirect ELISA. The purified IgG2b MAb was characterised by western blots and evaluated in sandwich-ELISA for reactivity against the homologous and heterologous antigens which include *Toxocara canis*, *Toxocara cati*, *Ancylostoma caninum* and *Ascaris suum*. An IgG2b MAb that recognises 30 kDa molecular weight proteins associated with strongyloidiasis and a cross-reaction with a 30 kDa for *Ancylostoma caninum* were observed. The IgG2b MAb was recognised by two *Strongyloides ratti* antigens and saline extract antigen of *Ancylostoma caninum* but did not react with other heterologous antigens in both assays. The antigen detection limit by sandwich ELISA was 75 ng/mL. Seventeen out of the twenty strongyloidiasis experimentally infected rat faeces (85%) evaluated for coproantigen using the IgG2b MAb have shown antigen-positive reactions in sandwich-ELISA. Similarly, only *Ancylostoma caninum* from dog faeces were reactive against the IgG2b MAb from all samples tested for cross-reactivity. This

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study concluded that the IgG2b MAb produced was able to detect Strongyloidiasis and Ancylostomiasis in animal models.

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

PENGHASILAN DAN UJIAN IMMUNODIAGNOSIS ANTIBODI MONOKLON 30 kDa TERHADAP KOPROANTIGEN DARI *Strongyloides* DALAM MODEL HAIWAN

Oleh

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Applikasi ujian diagnostik terhadap strongiloidiasis usus sukar dilakukan dan kaedah-kaedah parasitologi seperti Baermann atau kultur piring agar akan mengambil masa yang terlalu lama, juga melibatkan pengambilan spesimen berulang kali sebanyak tiga kali dilakukan pada jangka waktu yang berbeda bagi mendapatkan hasil yang sensitif dan tepat. Kaedah serologi yang digunakan sekarang ini untuk ujian diagnostik makmal terhadap strongiloidiasis usus pada manusia telah menunjukkan berlakunya tindakbalas silang dengan lain-lain jenis helmint gastrousus. Ini adalah merupakan kajian diperingkat awal, yang bertujuan untuk menghasilkan dan menilai antibodi monoklon terhadap koproantigen dari *Strongyloides ratti* menggunakan model haiwan penyelidikan. Model jangkitan *Strongyloides ratti* diwujudkan di dalam tikus (Sprague Dawley) yang imun tertindas. Larutan salin ekstrak protein dari larva infektif (iL3) telah digunakan sebagai antigen untuk proses imunisasi terhadap mencit BALB/c. Sel-sel limpa yang telah dituai dari mencit yang telah diimmunisasi akan digabungkan dengan sel-sel mieloma (SP2/0) untuk penghasilan hibridoma. Larutan supernatan dari hibrid yang telah dikenal pasti positif akan disaring dengan ELISA. Antibodi yang terhasil (IgG2b MAb) dicirikan melalaui kaedah pemendapan Western dan ditentukan dengan menggunakan ELISA-terapit bagi reaktiviti terhadap antigen homologus dan heterologous, termasuk dari *Toxocara canis*, *Toxocara cati*, *Ancylostoma caninum* dan *Ascaris suum*. IgG2b MAb dapat mengenalpasti 30 kDa berat molekul protein terhadap strongyloidasis serta menghasilkan tindakbalas silang dengan 30 kDa dari *Ancylostoma caninum*. Ini menunjukkan bahawa epitop tunggal dapat dikenal pasti oleh IgG2b MAb, justeru itu, boleh digunakan untuk tujuan ujian diagnostik. IgG2b MAb telah dkenal pasti dengan menggunakan dua jenis antigen dari *Strongyloides ratti* dan antigen dari salin ekstark protein dari *Ancylostoma caninum*, tetapi tidak bertindak balas dengan lain-lain jenis antigen yang heterologous menggunakan

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kedua-dua asai. Had mengesan antigen oleh ELISA-terapit adalah pada 75 ng/mL. Sebanyak tujuhbelas tinja tikus (85%) yang positif terhadap koproantigen telah dikesan oleh IgG2b MAb daripada dua puluh jumlah tinja tikus yang diuji menggunakan ELISA-terapit. Begitu juga, hanya tinja anjing yang disahkan positif dengan *Ancylostoma caninum* telah berjaya dapat dikesan oleh IgG2b MAb menggunakan ujian tindalbalas silang. Kesimpulannya, IgG2b MAb telah berjaya mengesan jangkitan Strongyloidiasis dan Ancylostomosis di dalam model haiwan penyelidikan.

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This thesis was submitted to the Senate of 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:

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LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cellular Cytotoxicity
ATCC	American Type Culture Collection
AF	Acid-fast
APC	Agar plate culture
APCs	Antigen presenting cells
BLAST	Basic local allignment search tool
BSA	Bovine serum albumin
Bp	Base pair
CDC	Centre for disease control
cDNA	Complementary de-oxyribonucleic acid
CE	Cystic echinococcosis
CFA	Complete Fruend's adjuvant
CGM	Complete general media
CLM	Cutaneous larva migrans
DMEM	Dulbeco's modified eagles medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylene-diamine-tetraacetic acid
EIA	Enzyme immunoassay
EPG	Egg per gram
ES	Excretory/secretory
FDA	Food and drug administration
FEA	Formalin-ethyl-acetate
FPSK	Faculti Perubatan dan Sains Kesihatan
GIT	Gastrointestinal tract
HAT	Hypoxanthine aminopterine thymidine
HGPRT	Hypoxanthine-guanine-phosphoribosyl-transferase

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HTLV-1	Human t-cell lymphotrophic virus 1
HRP	Horseradish peroxidase
HCl	Hydrochloric acid
IACUC	Institutional Animal Care and Use Committee
IB	Immunoblotting
IFA	Incomplete fruend's adjuvant
IFAT	Immunofluorescent antibody test
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgE	Immunoglobulin E
IL-2	Interleukin 2
L2	Second-stage larva
iL3	Third-stage (infective) larva
LPG	Larvae per gram
IF	Immunofluorescence
IRIS	Immune reconstitution inflammatory syndrome
ITS1	Internal transcribed spacer 1
INF-α	Interferon alpha
INF-ɣ	Interferon gamma
kDa	Kilodalton
LIPS	Luciferase immunoprecipitation system
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
MFFT	Modified faecal filteration techniques
ML	Muscular larva
NaOCl	Sodium hypochloride
NCBI	National Centre for Biotechnology Information

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NCM	Nitrocellulose membrane
OD	Optical density
PEG	Polyethylene glycol
PI	Post infection
pH	Hydrogen ion concentration
PBS-T	Phosphate buffered saline-tween
PCR-RFLP	Restriction fragment length polymorphism
qPCR	Quantitative polymerase chain reaction
RIA	Radio immuno assay
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
STH	Soil transmitted helminth
SE	Saline extract
TEMED	Tetramethylethylenediamine

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CHAPTER 1

INTRODUCTION

1.1 Background Information

The genus *Strongyloides* consists of more than 40 species of helminth parasites of human, bird, amphibian and reptile hosts, whereas the majority are parasites of domesticated mammals, including 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 environments. It is widespread in all tropical and subtropical countries of the world (Tiwari *et al*., 2012). However, it may also be present in temperate countries with favourable weather conditions. *Strongyloides stercoralis* infection can also be identified in non-endemic countries due to increase migration and travel.

The infection is however, more common in migrants than in travellers (Bethony *et al.*, 2006). *Strongyloides stercoralis* infection is similarly been reported to belong to the heterogeneous group of under-researched neglected tropical diseases (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). The parasitic worm is also present in some limited areas of the European countries such as northern Italy, rural Romania or south-eastern Spain.

Strongyloides stercoralis affects nearly 30 - 100 million people worldwide, but this could be an underestimation due to lack of affordable gold-standard diagnostic technique (Knopp *et al.*, 2009). However, little information is available about emerging consequences in endemic areas and the epidemiological data are still scarce in resource poor tropical countries. The higher suspicion index of *Stronyloides stercoralis* infection requires early accurate diagnosis and effective treatment (Becker *et al.*, 2011).

The infection is usually asymptomatic except in hyperinfection and disseminated type of the disease. 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.

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Clinically, peripheral eosinophilia may often be the first and only visible sign of *strongyloides* infection (Koczka *et al.*, 2012). Eosinophilia and rhabditiform larvae in the stool may be the only indicators of infection, but eosinophilia is not usually obligatory and larval detection is uncommon even after repeated sample examination (Azira & Zeehaida., 2010; Tiwari *et al*., 2012). Human infections are acquired by the filariform larvae (infective stage), through the sub-cutaneous route which penetrate the human skin and migrate into the sub-mucosa, then into the venous circulation, and then to the right heart and lungs (Tiwari *et al*., 2012).

Strongyloides stercoralis has a complicated life cycle, adult females are the parasitic stages which live embedded within the mucosa of the small intestines the adult female produced eggs to yield the rhabditiform larvae which are finally released into the lumen with stools. The characteristic feature of this parasite is its ability to persevere and propagate within its host (auto- infection cycle) for decades and this lead to infection with high death rates particularly in immunocompromised hosts (Segarra-Newnham., 2007).The hyperinfection syndrome is typically characterized by intensity and movement of the infective larvae to many organs and tissues including the lung and intestine (Agrawal *et al*., 2009; Anjos-Ramos *et al*., 2016). This phenomenon is what is referred to as disseminated infection and manifest in severe organ pathology and deaths (Lim *et al*., 2004).

Hyperinfection syndrome and disseminated infection occur more often among patients with immune suppressing diseases like Human T-cell lymphotropic viral disease, Acquired immunodeficiency syndrome, and patients with autoimmune disease or undergoing organ transplantation (Carvalho and Da Fonseca Porto., 2004; Patele *et al*., 2008; Keiser and Nutman., 2004; Krishnamurthy *et al*., 2007). Other conditions associated with immune suppression are congenital immunodeficiencies, as well as patients undergoing prolonged immunosuppressive therapy like cancer (Evering and Weiss., 2006; Fardet *et al*., 2007; Dall'Agnol *et al*., 2014).

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 considered a suitable alternative parasite to work with in the laboratory in rather than Strongyloides stercoralis. Experimental infection model of the rodent species of this nematode (Strongyloides ratti and Strongyloides venezuelensis) have been used to avoid the accidental risk of infectivity associated with the use of Strongyloides stercoralis. Strongyloides ratti has both direct and indirect life cycle, without any insect vector required to complete the cycle and like several other gastrointestinal nematodes, it follows the normal pulmonary cycle of development (Grove, 1989). 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).

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1.2 Statement of the Problem

Diagnosis of *Strongyloides stercoralis* relies on detection and identification of the eggs and rhabditiform larvae in the stool using a number of conventional techniques which includes direct faecal smear stained with Lugol iodine, Harada-Mori filter paper culture, Baermann concentration, and agar plate cultures. 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 reports of ≤ 25 rhabditiform larvae per gram (LPG) of faecal samples. It has been demonstrated that a single stool examination is insufficient to identify larvae in approximately two-third of the cases studied.

Repeated stool specimen examinations increase the probability of detecting the parasitic stages. Diagnostic sensitivity can approach 50-100% rise, if only about 3 to 7 serial stool samples are examined respectively (Pelletier., 1984; Nielsen & Mojon., 1987; Montes *et al.*, 2010). The peripheral blood eosinophil count is not an accurate indicator of parasitic infection. Eosinophilia is a common finding in the chronic stage of *Strongyloides stercoralis* patients, but in the immunocompromised individuals (patients receiving immunosuppressive therapy or with hyperinfection syndrome), the eosinophils count is unreliable (Al-Hasan *et al.*, 2007). This is because they usually show a decreased eosinophil 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). Serological diagnostic assays (ELISA, IFA) can simplify and overcome the poor sensitivity of *S. stercoralis* detection when single stool sample is examined.

These assays are usually employed 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) is an a sensitive alternative diagnostic tool which relies on the use of crude somatic antigen extract of *Strongyloides* species to measure IgG responses in the patient‘s serum. They are mostly applied for antibody detection but cannot distinguish between previous and active infection and this usually results in false positive results especially in acute infections (Krolewiecki *et al.*, 2010).

Furthermore, cross-reactivity was reported in patients with other related nematode parasitic infections which was attributed to similarity in most somatic antigenic epitopes been shared among them, including active filarial infections (Ramanathan & Nutman, 2008; Utzinger *et al.*, 2012). Antigen detection techniques are still lacking especially in serological assays while copro-antigen detection studies through sensitization of plates with polyclonal antibodies produced in rabbits have been used in the diagnosis of hyperinfection strongyloidiasis (Taweethavonsawat *et al.*, 2002; El-Badry, 2009). Molecular detection techniques like PCR have been reported to be both sensitive and specific, which is good (Verweij *et al.*, 2009) but expensive and time consuming, and almost unaffordable in most of the endemic areas due to poor resources settings.

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1.3 Significance of the Study

Improved methods for the diagnosis of strongyloidasis infection are necessary to develop a more rapid, reliable and affordable clinical diagnostic test reagent and to support prevalence estimates for disease prevention and control (Segarra- Newnham, 2007). Diagnostic techniques for strongyliodasis must be highly sensitive and specific for detecting active infection. They should be of low cost and available to use in the area where the disease is prevalent (Dekoumyoy *et al.*, 2002).

There is also an urgent need to overcome the short-comings associated with the conventional detection techniques for effective diagnosis and treatment of the condition in high risk individuals before the unset of immunosuppression of any kind. **S**tudies to use specific monoclonal antibodies for copro-antigen detection in intestinal strongyliodasis prior to hyperinfection and dissemination has been based on the fact that eggs and/rhabditiform larvae are the only stages detectable in faeces. There may be a possibility that antigens from these two stages could be easily detected from stool samples.

1.4 Research Hypothesis

- Soluble larval homogenate protein (saline extract) antigen from the infective larvae (iL3) of *Strongyloides ratti* can stimulate immune response for the production of specific antibodies.
- The monoclonal antibodies will bind with the epitope of coproantigen from *Strongyloides ratti* in immunodiagnostic application.

1.5 General Objective

To develop monoclonal antibody against the antigen of the infective larvae of *Strongyloides ratti* for use in immunodiagnosis of active intestinal strongyloidiasis.

1.5.1 Specific Objectives

1. To establish *Strongyloides ratti* biological animal model for preparation of antigen.
2. To develop and characterize monoclonal antibody against the soluble larval (iL3) homogenate protein antigen of *Strongyloides ratti.*
3. To determine the cross reaction of the candidate monoclonal antibody (MAb) against other related parasite antigens.
4. To evaluate the candidate monoclonal antibody for detecting coproantigens in faeces of *Strongyloides ratti* infected rats based on crossreaction with heterologous antigen.

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