UNIVERSITI PUTRA MALAYSIA

PRODUCTION AND EVALUATION OF MONOCLONAL ANTIBODY AGAINST Strongyloides ratti

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By

MAHMUDA ALIYU

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

March 2017

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DEDICATION

This research work is dedicated with all humility and respect to my beloved parents for their love, guidance, support and patience.

Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PRODUCTION AND EVALUATION OF MONOCLONAL ANTIBODY AGAINST *Strongyloides ratti*

By

MAHMUDA ALIYU

March 2017

Chairman : **Ngah Zasmy AL/Unyah, PhD**
Faculty : **Medicine and Health Sciences**

Currently, most of the available serological diagnostic kits for strongyloidiasis are based on the application of the crude antigens of *Strongyloides ratti*, which are good, but with less sensitivity for diagnosis of active infection. Hence this study is aimed to produce and evaluate monoclonal antibody for detecting soluble parasite antigen in animal sera prior to hyperinfection and disseminated strongyloidiasis. *Strongyloides ratti* infection model was established and maintained in immunosuppressed Sprague Dawley rats. Saline extract protein from the infective larvae (iL3) of *Strongyloides ratti* was used to immunise BALB/c mice. The B-lymphocytes from the spleen taken from the immunised mice were fused with myeloma (SP2/0) cells using 50% polyethylene glycol (PEG) for somatic cell hybridization. The hybridomas were cultured in hypoxantine-aminopterin-thymidine (HAT) medium and cloned by limiting dilutions. Supernatants from the growing positive hybrids were screened by indirect ELISA using 96-well plates coated with the saline extract-protein. The ascites fluid induced by intraperitoneal injection of the antibody-secreting hybridoma cells was purified by a MAb IgG purification kit. The purified antibody (MAb) was characterised by western blots and evaluated in capture ELISA for reactivity against the homologous and heterologous antigens of *Ascaris suum*, *Toxocara canis*, *Ancylostoma caninum* and *Toxoplasma gondii*. An IgG1 MAb that recognises 30 kDa and 34 kDa associated with strongyloidasis and a cross-reaction with a 30 and 34 kDa for toxocariasis were observed. This indicates that more than one epitope is recognised by the MAb, thus, making it valuable for diagnostic purpose. The MAb was recognised by all *Strongyloides ratti* antigens and *Toxocara canis* antigens but did not react with other heterologous antigens in both assays. From the results obtained using the saline extract protein antigen concentration standard curve, it is confirmed that the antigen detection limit by sandwich ELISA was 5 ng/mL, which provides sufficient sensitivity for the diagnosis of Strongyloidiasis. All twelve (12)

strongyloidiasis infected rat sera evaluated for circulating antigen using the MAb produced, have shown antigen-positive reactions in sandwich ELISA. Similar results were obtained from Toxocara infected animal sera. This study concluded that the MAb produced was able to detect strongyloidiasis and toxocariasis in animal models and may also be used for serological diagnosis of strongyloidiasis and toxocariasis in human sera.

Abstrak thesis yang dikemukakan kepada Senate Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN DAN PENILAIAN ANTIBODI MONOKLON TERHADAP *Strongyloides ratti*

Oleh

MAHMUDA ALIYU

Mac 2017

Pengerusi : Ngah Zasmy Unyah, PhD
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Pada masa ini, kit-kit ujian diagnostik serologi untuk strongiloidiasis kebanyakannya adalah berdasarkan kepada aplikasi antigen-antigen kasar dari *Strongyloides ratti*, kit-kit tersebut sememangnya boleh digunakan, walau bagaimanapun, kit-kit tersebut mempunyai sensitiviti yang rendah terhadap jangkitan aktif. Oleh itu, kajian ini adalah untuk menghasilkan dan menilai antibodi monoklon bagi tujuan mengesan antigen parasit terlarut di dalam sera haiwan sebelum berlakunya jangkitanhiper dan strongiloidiasis tersebar. Model jangkitan *Strongyloides ratti* telah berjaya dihidupkan dan dikekalkan di dalam tikus Sprague Dawley. Protein ekstrak salin dari larva infektif (iL3) dari *Strongyloides ratti* telah digunakan untuk mengimunkan mencit BALB/c. Sel-sel B-lymphocyte yang di keluarkan dari limpa mencit yang telah terimun, disatukan dengan sel-sel mieloma (SP2/0) bagi tujuan penghibridan sel-sel somatik dengan bantuan polyethylene glycol (PEG) sebanyak 50% dari kepekatan asal. Medium hypoxantine-aminopterin-thymidine (HAT) digunakan untuk pengkulturan sel-sel hibridoma dan diklonkan dengan kaedah pencairan pengehad. Supernatan dari kacukan hibrid positif yang membangun akan di saring dengan ELISA menggunakan plat 96-berperigi yang telah disaluti dengan protein ekstrak salin. Cecair asites yang terhasil dari suntikan intraperitoneum dengan sel-sel hibridoma perembes-antibodi yang telah ditulenkan menggunakan kit penulenan Mab IgG. Antibodi tertulen (MAb) telah dicirikan dengan kaedah blots western dan dinilaikan menggunakan ELISA tertangkap untuk melihat reaktiviti terhadap antigen-antigen homologus dan heterologus seperti *Ascaris suum*, *Toxocara canis*, *Ancylostoma caninum* dan *Toxoplasma gondii*. IgG1 MAb yang dikenal pasti pada 30 kDa dan 34 kDa adalah berkait rapat dengan strongyloidiasis dan tindak balas silang dengan 30 kDa and 34 kDa juga telah dikenal pasti. Ini menunjukkan bahawa lebih daripada satu epitope dapat dikesan oleh MAb, oleh itu, ini menjadikan ia penting khusus untuk tujuan diagnostik. MAb telah dapat mengesan *Strongyloides ratti* dan

Toxocara canis tetapi tidak dapat bertindak balas dengan antigen heterologus yang lain pada kedua-dua jenis assai. Dari keputusan yang telah diperolehi dengan menggunakan lengkungan kepekatan piawai, ini mengesahkan bahawa had pengesanan antigen oleh ELISA berapit adalah pada 5 ng/mL, yang daapt menyediakan kepekaan yang mencukupi bagi tujuan diagnosis strongyloidiasis. Kesemua dua belas (12) sera tikus yang telah dijangkiti strongyloidiasis bagi tujuan mengesan kehadiran antigen beredar *Strongyloides ratti* telah menunjukkan reaksi antigen-positif di dalam ELISA berapit. Keputusan yang sama juga dapat dilihat pada sera haiwan yang telah dijangkiti dengan *T. canis.* Kajian ini menyimpulkan bahawa MAb yang telah terhasil dapat mengesan kedua-dua jangkitan, iaitu, strongyloidiasis dan toksokariasis dalam model haiwan pada kajian yang telah dijalankan, ini juga mungkin dapat digunakan untuk diagnosis serologi terhadap strongyloidiasis dan toksokariasis yang menggunakan sera manusia.

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I certify that a Thesis Examination Committee has met on 13 March 2017 to conduct the final examination of Mahmuda Aliyu on his thesis entitled "Production and Evaluation of Monoclonal Antibody Against *Strongyloides ratti*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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Name of Member of Supervisory Committee: Associate Professor Dr. Rukman Awang Hamat

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ATCC	American Type Culture Collection
ATL	associated adult t-cell leukemia
APCs	antigen presenting cells
Bcc	*Burkholderia cepacia* complex
BLAST	basic local allignment search tool
BSA	bovine serum albumin
CBPP	contagious bovine pleuropneumonia
CDC	Centre for Disease Control
CD4	cluster of differentiation 4
cDNA	complementary de-oxyribonucleic acid
CE	cystic ecchinococcosis
CFA	complete fruend's adjuvant
CLM	cutaneous larva migrans
DMEM	dulbeco's modified eagles medium
DMSO	dimethyl sulfoxide
EDTA	ethylene-diamine-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPG	egg per gram
ES	excretory/secretory
FDA	Food and Drug Administration
FPSK	Faculti Perubatan dan Sains Kesihatan
HAT	hypoxanthine aminopterine thymidine
HGPRT	hypoxanthine-guanine-phosphoribosyl-transferase
HTLV-1	human t-cell lymphotrophic virus 1
HSCT	hemapoetic stem cell transplant
HRP	horseradish peroxidase
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
IHC	immunohistochemistry
IL-2	interleukin 2
IRIS	immune reconstitution imflammatory syndrome
ITS1	internal transcribed spacer 1
INF-α	interferon alpha
INF-ɣ	interferon gamma
kDa	kilodalton

LAMP	loop-mediated isothermal amplification
LAT	latex agglutination test
LDL-C	low-density lipoprotein
LPS	lipopolysaccharide
LIPS	luciferase immunoprecipitation system
MAb	monoclonal antibody
MAT	modified agglutination test
MHC	major histocompatibility complex
MFFT	modified fecal filtration technique
NaOCl	sodium hypochloride
NCBI	National Centre for Biotechnology Information
OD	optical density
PCR-RFLP	polymerase chain reaction-restriction fragment length polymorphism
PEG	polyethylene glycol
PI	post infection
PVDF	polyvinylididine difluoride
Psa	pneumococcal surface adhesin
pH	hydrogen ion concentration
PBS-T	phosphate buffered saline-tween
RIA	radio immuno assay
RIT	radio-immune theraphy
RNA	ribonucleic acid
RPM	revolutions per minute
RPMI	Roswel's Park Memorial Institute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
STH	soil transmitted helminth
SE	saline extract
SSIR	strongyloides stercoralis immunoreactive
TCS	tissue culture supernatant
TEMED	tetramethylethylenediamine
Th1	t-helper 1
Th2	t-helper 2

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Strongyloidiasis is an intestinal parasitic infection of humans that can be manifested as a chronic infection, hyperinfection or a disseminated disease (Ghoshal *et al*., 2012). The disease has a world-wide distribution but is common and has a prevalence of 30 - 40% of the total population in tropical and sub-tropical countries from African, Asia and South America (Vilela *et al*., 2009). Immunocompetent individuals usually have asymptomatic infections which are characterised by eosinophilia only (Mir *et al*., 2006). Human infection is generally acquired through skin panatration by filariform larvae which then develop to gastrointestinal and pulmonary clinical signs (Ardiç, 2009). *Strongyloides stercoralis* is the only human parasitic nematode that is able to persist and replicate within its host (auto-infection cycle) for decades and this lead to infection with high mortality rates especially in immunocompromised hosts (Segarra-Newnham, 2007).

The auto-infection cycle results in a syndrome called hyperinfection which is characterised by intensive invasion and migration of the infective larvae in the lungs and intestines as a result of increase burden due to immunosuppressive conditions in infected patients (Agrawal *et al*., 2009; Anjos-Ramos *et al*., 2016). This hyperinfection syndrome results in severe organ pathology and subsequent death due to dissemination of larvae into multiple organs (Lim *et al*., 2004), but early detection of the infection may alter the fatal course of the disease through proper and timely treatment. Infective larvae may also penetrate the gastrointestinal tract around the colon which can lead to sepsis and multiple organ failure as a result of migration of enteric pathogens along the larval tracts and further symptoms associated with dissemination (Boulware *et al*., 2007).

Hyperinfection and dissemination are regularly observed in immunocompromised individuals such as patients with Human T-cell Lymphotropic Viral disease (Carvalho and Da Fonseca Porto, 2004), after organ transplantation (Patel *et al*., 2008), and has also been reported in patients with Acquired Immunodefficiency Syndrome (Keiser and Nutman, 2004). Patients with autoimmune diseases (Krishnamurthy *et al*., 2007), congenital immunodeficiencies (Evering and Weiss, 2006), as well as patients under prolonged immunosuppressive therapy for asthma and other inflammatory conditions are high risk individuals (Fardet *et al*., 2007; Dall'Agnol *et al*., 2014).

Diagnosis of infections with *Strongyloides stercoralis* is usually done by examination of host's feces for the presence of eggs/rhabditiform larvae or by coprocultures for the detection of filariform larvae (Knopp *et al*., 2014). Except for hyper-infections and disseminated disease finding, finding eggs in feces has always been very difficult due to the autoinfection cycle. Furthermore, this also affects the number of larvae found during fecal cultures (Habtamu *et al*., 2011). This phenomenon affects the sensititivity of the various conventional methods of diagnoses, thereby leading to negative results even after repeated sample examinations. Molecular detection techniques like PCR have been reported to be both sensitive and specific (Verweij *et al*., 2009) while serological techniques (ELISA, IFA) were reported to be sensitive but with extensive cross reactivity with related nematode parasites (Utzinger *et al*., 2012).

Laboratory diagnosis of Strongyloidiasis can be done using commercially available ELISA kits based on antigens extracted from *S. ratti*. However most of the kits will detect antibodies (Abnova®, Bordier®, AccuDiag™, NovaLisa®, and SCIMEDX etc). The use of crude extract from the filariform larvae of *S. ratti* as antigen in ELISA has always yielded a false positive results (cross reactivity) with sera from patients infected with other helminth parasites (Conway *et al*., 1993; Sultana *et al*., 2012; Ahmad *et al*., 2013). Thus, it is expected that the use of monoclonal antibodies specific to the parasite antigen (protein) would offer high specificity and sensitivity to the serological diagnosis of the disease (Siddiqui and Berk, 2001).

In the absence of early diagnosis of strongyloidiasis, the prognosis of disseminated disease is extremely poor but treatment is usually effective when infections are detected early. It is essential that physicians be aware of the diagnostic shortcomings especially when dealing with immunocompromised patients and the importance of ruling out strongyloidiasis before immunosuppressive regimens should be considered.

In vivo maintenance of *Strongyloides stercoralis* has been done in gerbils (*Meriones unguiculatus*) and dogs as a laboratory hosts for research purpose (Shariati *et al*., 2010). However, this can only be achieved if sufficient quantities of infective larvae (iL3) of *Strongyloides stercoralis* are successfully isolated from an infected human patient. This process/technique posses ethical concern and can be tedious and time consuming (Nolan *et al*., 2002). On the other hand, large quantities of infective larvae (iL3) of the rodent species, *Strongyloides ratti* and *Strongyloides venezuelensis* can be easily isolated from naturally infected wild rats (*Rattus* spp) and maintained in rats as laboratory model for research.

Interestingly, *Strongyloides ratti* is a commonly found helminth parasites in wild rodents and it is morphologically and phylogenetically closely related to *Strongyloides stercoralis* which infect humans (Dorris *et al*., 2002; Matsuda *et al*., 2003). Due to these related similarities, an extract from *Strongyloides*

ratti has been used as an antigen in immunodiagnoses of human strongyloidiasis research (Rodrigues *et al*., 2004, 2007).

1.2 Statement of the problems

Human strongyloidiasis is a potentially life threatening parasitic infection especially in immunocompromised individuals. Diagnosis using conventional methods (direct smear, formol-ether concentration) usually lacks sensitivity due to inconsistent presence of parasite eggs and/or rhabditiform larvae in the fecal samples of majority of patients, even after repeated sample examination from same infected individual (Inês *et al*., 2011). Invasive methods such as duodenal intubation (Agrawal *et al*., 2009) and biopsy may be used to collect sample specimens which are then subjected to parasitological methods of diagnosis (Baemann's technique and culture), and have been reported to be more sensitive than the conventional fecal smearing method. However, the use of these procedures is very unpleasant to patient, which prevents their routine application (Lemos *et al*., 2003).

Diagnosis by antibody detection (ELISA) has been hampered by lack of specificity as a result of cross-reaction with related helminth parasites. Most available ELISA kits for the diagnosis of strongyloidiasis are based on detection of specific antibodies but not antigen. Thus, they are unable to differentiate active form previously acquired infections and often lead to false positive test results in sero-converted patients. Diagnosis using antigen detection in feces has been reported to be sensitive only in hyperinfection cases but not in chronic infections. Sensitivity and specificity of the serological techniques vary depending on the type of antigen used among many other attributes (Taweethavonsawat *et al*., 2002). Molecular techniques (PCR) are sensitive and specific but have been reported to be very expensive, time consuming (Levenhagen and Costa-Cruz, 2014), and almost not available in most endemic areas due to poor resource settings. As such, alternative methods have been sought for accurate and rapid diagnosis of Strongyloidiasis.

1.3 Justification of the research

There is no doubt that improved methods for the diagnosis of active strongyloides infection is needed to support clinical diagnosis of the disease and to determine recent prevalence for disease monitoring and control (Segarra-Newnham, 2007). Ideal diagnostic test for strongyloidiasis should have a high sensitivity and specificity for detecting active infection. They should be cheap and applicable for use in the countries where the disease exists (Dekumyoy *et al*., 2002). This necessitates the continued search for more reliable approaches and robust techniques that would offer high specificity for an accurate and rapid diagnosis of active Strongyloidiasis.

Our approach to diagnosing Strongyloidiasis using monoclonal antibody is based on the assumption that because *S*. *stercoralis*, being relatively the smallest nematode parasite that are carried through the circulatory system and spend most of its life cycle within the tissues of the infected host, will release some amounts of antigens in the blood of the host individual. Thus, one ought to detect traces of these parasites or its antigen in the blood of the infected host (parasitemia). It is hoped that these monoclonal antibodies can be used to improve the sensitivity/specificity of capture ELISA for antigen detection as they have been proven effective as a result of their specific-binding ability to target antigens and to overcome the problems associated with the conventional detection assays.

1.4 Research hypotheses

- Saline extract antigen from the infective larvae (iL3) of *Strongyloides ratti* can stimulate in mice an immune response that will be used for the production of monoclonal antibodies specific for strongyloides antigens.
- The monoclonal antibodies specific for antigens of *Strongyloides ratti* can be used to detect strongyloides antigens in sera of infected animals.

1.5 General objective

To develop monoclonal antibodies against *Strongyloides ratti* antigen for use in immunodiagnosis of active Strongyloidiasis in an experimental animal model.

1.5.1 Specific objectives

1. To establish and maintain a *Strongyloides ratti* infection model and to prepare saline extract protein from infective larvae (iL3) for immunisation of mice.
2. To develop and characterize monoclonal antibodies against the saline extract protein of *Strongyloides ratti*.
3. To determine the specificity of the candidate monoclonal antibody (MAbs) against other related parasite antigens.
4. To evaluate the candidate monoclonal antibody in capture ELISA for detecting circulating antigens in sera of *Strongyloides ratti*-infected rats.

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