CLONING, EXPRESSION AND PURIFICATION OF *Toxocara canis* RECOMBINANT ANTIGENS (rTES-26, rTES-32, rTES-120) AND DEVELOPMENT OF SERODIAGNOSTIC TEST FOR TOXOCARIASIS

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by

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DEDICATIONS

To

My husband, Mohd Rozi Aziz,

My mother, Nik Hanizan and my mother-in-law, Zainab,

My father, Mohamad and my father-in-law, Aziz

My sons, Mohamad Rasydan Hakimi, Mohamad Rafsyan Hakim, Mohamad Rahaizat Hakimin and Mohamad Raqwan Hatim

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LIST OF ABBREVIATIONS & ACRONYMS

µg	microgram
µl	microliter
ABTS	2,2'-Azino-bis(3-ethylbenthiazoline-6- sulphonic acid)
AEC	absolute eosinophil counts
AP	ammonium persulfate
BM	Boehringer Mannheim
bp	base pair
CFT	complement fixation test
CIEP	countercurrent immunoelectrophoresis
CT	covert toxocariasis
CV	column volume
DEC	diethylcarbamazine
DEPC	diethyl pyrocarbonate
DFAT	direct fluorescent antibody tests
DGDT	double gel diffusion test
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EME	eosinophilic meningo-encephalitis
ES	excretory secretory
FPLC	Fast Protein Liquid Chromatography
GST	glutathione S-transferase
GWC	Goldmann-Witmer coefficient
HRP	horseradish peroxidase
HUKM	Hospital Universiti Kebangsaan Malaysia
HUSM	Hospital Universiti Sains Malaysia
IACE	indirect antibody competition ELISA
ICT	immunochromatography test
IFAT	indirect fluorescent antibody test
IL	interleukin

IPTG	isopropyl- ß-D- thiogalactopyranoside
K_2HPO_4	dipotassium hydrogen phosphate
kb	kilobase
kDa	kilo dalton
KH_2PO_4	potassium dihydrogen phosphate
L2	stage two larva
LB	Luria-Bertani
MCS	multiple cloning site
mg	milligram
$MgCl_2$	magnesium chloride
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MtDNA	mitochondrial DNA
N_2	nitrogen
Na_2HPO_4	disodium hydrogen phosphate
NaH_2PO_4	sodium dihydrogen phosphate
$NaHCO_3$	sodium bicarbonate
Ni^{2+}	níckel ion
NIH	National Institutes of Health
Ni-NTA	nickel - nitrilo-tri-acetic acid
nm	nanometer
NPV	negative predictive value
NS	nephrotic syndrome
NTD	neglected tropical diseases
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	PBS-Tween 20
PCR	polymerase chain reaction
pmol	picomole
POD	peroxidase
PPV	positive predictive value
PRIST	paper radio-immunosorbent test
RAPD	random amplification of polymorphic DNA
RAST	radio-allergosorbent test
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RIA	radio immunoassay
RNA	ribonucleic acid
rpm	revolution per minute
RPMI	Roswell Park Memorial Institute
rTES	recombinant *Toxocara* excretory-secretory
RT-PCR	reverse-transcriptase polymerase chain reaction
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
SOC	Super Optimal broth with Catabolite repression
STH	Soil-transmitted helminthiases
TB	Terrific Broth
TBE	Tris-Borate EDTA
TBS	Tris buffered saline
TBS-T	TBS-Tween 20
TBSTT	TBS/Tween 20/Triton-X
TEMED	N,N,N',N'-Tetramethylethylenediamine
TES	*Toxocara* excretory-secretory

Th2	T helper 2
TMB	3,3',5,5'-tetramethylbenzidine
USB	ultrasound biomicroscopy
UV	ultra violet
VLM	visceral larva migrans
WB	Western blot

PENGKLONAN, PENGEKSPRESIAN DAN PENULENAN ANTIGEN REKOMBINAN *Toxocara canis* (rTES-26, rTES-32, rTES-120) DAN PEMBANGUNAN UJIAN SERODIAGNOSTIK BAGI TOKSOKARIASIS

Abstrak

Serodiagnosis rutin untuk penyakit toksokariasis pada manusia adalah berdasarkan kit ELISA-IgG tak-langsung yang menggunakan antigen ekskretori-sekretori natif daripada *Toxocara canis*. Walau bagaimanapun, asai tersebut mempunyai spesifisiti yang rendah, terutamanya untuk kegunaan di negara tropika yang mempunyai pelbagai jangkitan parasit. Dalam usaha untuk meningkatkan kejituan ujian diagnostik bagi penyakit ini, asai ELISA-IgG4 telah dibangunkan dengan menggunakan tiga antigen rekombinan.

Dalam kajian ini, DNA rekombinan *T. canis* yang mengkodkan antigen rekombinan rTES-26, rTES-32 dan rTES-120 dihasilkan dengan mengklonkan kerangka bacaan terbuka gen masing-masing melalui kaedah "reverse-transcriptase-PCR" (RT-PCR) menggunakan mRNA yang diekstrak daripada kultur larva *T. canis* peringkat kedua ke dalam vektor PCR2.1 TOPO. Analisis jujukan menunjukkan TOPO/TES-32 and TOPO/TES-120 mempunyai persamaan 100% dengan jujukan yang dilaporkan dalam "GenBank", tetapi fragmen gen TOPO/TES-26 mempunyai empat mutasi. Selepas kesemua mutasi dalam TOPO/TES-26 diperbaiki, TES-26 dan TES-32 kemudiannya disubklonkan ke dalam vektor

ekspresi yang mempunyai penanda GST, manakala TES-120 disubklonkan ke dalam vektor yang mempunyai penanda histidin, dan kesemuanya diekspreskan di dalam hos ekspresi *E. coli* BL21(DE3).

Protein rekombinan tersebut kemudiannya ditulenkan secara natif dengan kaedah kromatografi afiniti menggunakan resin GST dan His-Trap kerana protein rekombinan tersebut banyak terhasil dalam bentuk yang terlarut. Protease tapak spesifik, Factor Xa, digunakan untuk membuang penanda GST dalam protein rekombinan TES-26 (rTES-26) dan TES-32 (rTES-32). Analisis blot Western menunjukkan antigen rekombinan tersebut adalah reaktif dan spesifik secara imunologik. Serum daripada pesakit toksokariasis yang mempunyai antibodi IgG4 dapat mengenalpasti antigen rekombinan tersebut, manakala serum daripada pesakit jangkitan lain dan individu sihat adalah tidak reaktif.

Apabila ketiga-tiga antigen rekombinan diuji dengan ELISA yang spesifik untuk immunoglobulin klas IgM dan IgE dan subklas IgG (IgG1-IgG4), keputusan jelas menunjukkan bahawa hanya asai IgG4 memberikan spesifisiti yang baik. Keupayaan diagnostik setiap antigen rekombinan tulen dan rTES-30USM (dihasilkan sebelum ini di dalam makmal kami) seterusnya dinilai dengan asai ELISA-IgG4 menggunakan sampel serum sebanyak 242 yang termasuk 30 pesakit toksokariasis dengan bukti klinikal, hematologi dan serologi. Kedua-dua asai rTES-26 dan rTES-32 ELISA menunjukkan sensitiviti 80.0%; manakala rTES-120 ELISA-IgG4 menunjukkan sensitiviti 93.3.0%, sama seperti yang telah dilaporkan sebelum ini untuk rTES-30USM ELISA-IgG4. Tahap sensitiviti rTES-120/rTES-30USM ELISA-IgG4 adalah lebih tinggi secara signifikan daripada rTES-26/ rTES-32 ELISA-IgG4 ($p<0.001$). Walau bagaimanapun, perbandingan min O.D 30 sampel toksokariasis di antara asai IgG4 dengan menggunakan empat antigen recombinan tidak menunjukkan perbezaan yang signifikan ($p>0.05$). Pada tahap signifikan $p<0.05$, tiada perbezaan yang signifikan di antara spesifisiti rTES-26 dan rTES-120 ($p=0.059$), di antara rTES-26 dan rTES-30USM atau di antara rTES-30USM dan rTES-120.

Dalam asai terakhir, rTES-32 tidak dimasukkan kerana ia tidak menunjukkan sensitiviti dan spesifisiti yang lebih baik daripada rTES-26. Malah rTES-30USM dimasukkan kerana sensitivitinya yang tinggi dan pengesanan 100% kes toksokariasis dapat dicapai apabila keputusan asai IgG4 menggunakan rTES-30USM dan rTES-120 digabungkan.

Kesimpulannya, asai terakhir yang sensitif (80.0% - 93.3%) dan spesifik (92.0% -96.2%**)** untuk pengesanan penyakit toksokariasis telah berjaya dibangunkan dengan menggunakan tiga telaga yang bersebelahan, setiap satunya disalut dengan rTES-26, rTES-30USM dan rTES-120. Kajian ini ádalah novel dalam beberapa aspek iaitu ia adalah yang pertama melaporkan penggunaan pelbagai antigen rekombinan untuk serodiagnosis toksokariasis; penggunaan rTES-26 (dan rTES-32) dalam serodiagnosis jangkitan toksokara; penggunaan asai IgG4 untuk rTES-120 dan rTES-26 dan penggunaan penanda GST dalam ekspresi dan penulenan protein recombinan toksokara. Ujian ini mungkin merupakan pembaharuan yang signifikan berbanding ujian komersial yang ada bagi diagnosis penyakit toksokariasis, terutamanya bagi negara yang ko-endemik dengan cacing tularan tanah yang lain.

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Abstract

Routine serodiagnosis of human toxocariasis is based on indirect IgG-ELISA kits which employ native *Toxocara canis* excretory-secretory (TES) antigen. However, these assays lacked specificity especially when used in tropical countries where multiparasitism are prevalent. In an effort to improve the diagnostic test for this infection, we have developed an IgG4-ELISA assay which uses three recombinant antigens.

In this study, recombinant *T. canis* DNA which encode for rTES-26, rTES-32 and rTES-120 were produced by cloning of open-reading frames (ORF) of the respective genes *via* reverse-transcriptase-PCR (RT-PCR) using mRNA extracted from a culture of *T. canis* second stage larvae into PCR2.1 TOPO vector. Sequence analysis revealed that TOPO/TES-32 and TOPO/TES-120 were 100% similar to the reported sequences in the GenBank, however, TOPO/TES-26 gene fragment had four mutations. After all mutations in TOPO/TES-26 gene fragments had been corrected, TES-26 and TES-32 were subsequently subcloned into a GST-tagged, while TES-120 was subcloned into a His-tagged prokaryotic

expression vector; and all constructs were expressed in *E. coli* BL21(DE3) expression host.

The recombinant proteins were subsequently purified under native condition by affinity chromatography using GST and His-Trap resins since these recombinant proteins were abundantly expressed in soluble form. The site-specific protease, Factor Xa, was used to remove GST tag in the TES-26 and TES-32 GST-tagged fusion proteins. Western blot analysis revealed that these recombinant antigens were immunologically reactive and specific. Sera from patients infected with toxocariasis had IgG4 antibodies that recognized these recombinant antigens, while sera from individuals with other infections and from healthy normals did not.

When the three recombinant antigens were tested in ELISAs specific for immunoglobulin IgM and IgE classes, and IgG subclasses (IgG1-IgG4), the results clearly showed that only IgG4 assay displayed good specificity. The diagnostic utility of each purified recombinant antigen and rTES-30USM (previously produced in our laboratory) was further evaluated by IgG4-ELISA assay using 242 serum samples which included 30 sera from patients with clinical, haematological and serological evidence of toxocariasis. Both rTES-26 and rTES-32 IgG4-ELISAs demonstrated sensitivity of 80.0%; while rTES-120 IgG4-ELISA showed sensitivity of 93.3.0%, which is similar to that previously reported for rTES-30USM IgG4-ELISA. The sensitivity of rTES-120/rTES-30USM IgG4-ELISA was found to be significantly higher than rTES-26/rTES-32 IgG4-ELISA ($p<0.001$). However, the mean O.Ds of the 30 toxocariasis samples among the IgG4 assays using the four recombinant antigens were shown not to be significantly different ($p>0.05$). At $p<0.05$, there was marginally no significant difference between the specificities of rTES-26 and rTES-120 ($p=0.059$), rTES-26 and rTES-30USM or between rTES-30USM and rTES-120.

In the final assay, rTES-32 was excluded since it was not better than rTES-26 in terms of sensitivity or specificity. Instead rTES-30USM was included due to its high sensitivity and the fact that a 100% detection of toxocariasis cases was achieved when results of IgG4 assays using rTES-30USM and rTES-120 were combined.

In summary, a final assay which is sensitive (80% to 93.3%) and specific **(**92.0%-96.2%**)** for detection of toxocariasis was successfully developed using three adjacent wells, each separately coated with rTES-26, rTES-30USM and rTES-120. This study is novel in several ways namely it is the first report of the use of multiple recombinant antigens for serodiagnosis of toxocariasis; the use of rTES-26 (and rTES-32) in *Toxocara* serodiagnosis; the use of IgG4 assay for rTES-120 and rTES-26 and the use of GST tag in the expression and purification of *Toxocara* recombinant proteins. These test maybe a significant improvement over commercially available tests for diagnosis of toxocariasis and may be use especially in countries co-endemic with other soil-transmitted helminthes.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Human toxocariasis is a worldwide parasitic zoonosis, caused most commonly by the parasite dog intestinal roundworm (*Toxocara canis*) and less frequently the cat roundworm (*Toxocara cati*) (Despommier, 2003; Fisher, 2003). Although several nematodes have been reported to produce visceral larva migrans (VLM), *T. canis* appears to be the primary causative agent (Glickman & Schantz, 1981). *T. canis* is more important in causing human infection than *T. cati* because dogs are more often in direct contact with people than cats. In addition cats usually select sandy soil for defecation and habitually bury *Toxocara* eggs-contaminated faeces, making infectious eggs less accessible to susceptible individuals (Overgaauw, 1997; Smith & Rahmah, 2006). Nevertheless, *T. cati* has been implicated particularly in ocular toxocariasis and represents an underestimated zoonotic agent (Fisher, 2003). Human toxocariasis is still a poorly diagnosed disease, especially in places with low socioeconomic level, and is largely unknown to health professionals and the general population (Wells, 2007). Nevertheless, it is probably one of the most common zoonotic helminthiases in temperate climates (Schantz, 1989).