UNIVERSITI PUTRA MALAYSIA

EXPRESSING THE GENE ENCODING 34-KILODALTON OUTER MEMBRANE PROTEIN OF Brucella melitensis FOR DEVELOPMENT OF A RECOMBINANT VACCINE

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the degree of Master of Veterinary Science

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Chairman : Professor Mohd. Zamri Saad, DVM, PhD

Faculty : Veterinary Medicine

Brucella is a Gram-negative, facultative intracellular bacterium that causes severe disease in both humans and animals. Brucellosis is still endemic in many developing countries, impairing animal health and productivity leading to important economic losses. *Brucella melitensis* causes abortions in sheep, goats, and cattle, and it is considered the most pathogenic *Brucella* spp. in humans. Currently, vaccination has been accepted as the best mean of control for brucellosis in small ruminant, with the aim at decreasing the prevalence of the disease to an acceptable level. Among available vaccines, *Brucella melitensis* Rev. 1, an attenuated smooth strain used to control *Brucella melitensis* infection in small ruminants, gives heterologous protection against *Brucella ovis* and is currently considered the best vaccine for the prophylaxis of brucellosis in sheep and goats. Yet Rev 1 has several disadvantages, namely residual virulence able to induce abortion in pregnant animals; a capacity to elicit antibodies against

smooth lipopolysaccharide (S-LPS) which interferes in the differential diagnosis between vaccinated and naturally infected animals; it is resistant to streptomycin, one of the antibiotics of choice used to treat brucellosis; it is pathogenic for humans; and its use is prohibited in countries free of *Brucella melitensis.* These several drawbacks indicate the need for a better vaccine for brucellosis eradication.

This study was conducted to analyze the outer membrane protein (Omp) profile and determine the antigenicity of local isolates of *Brucella melitensis*, followed by development of recombinant cells expressing the selected Omp and finally, study the immune response following exposure to the recombinant cells. The Omps of local isolates of *Brucella melitensis* strains 152, 183, and 293 were extracted using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) producing three major and four minor Omp bands, which were common in all isolates. They were the 25, 34 and 89 kDa major bands and the 16, 30, 43 and 70 kDa minor bands. Subsequent immunobloting identified consistently common antigenic bands that were shared by all strains. They were the 16, 34 and 70 kDa bands. The 34 kDa was the only major Omp band found to be antigenic.

Investigations at the molecular level involved isolation and detection of the gene encoding 34 kDa Omp of *Brucella melitensis* strain 293. As a result from the cloning procedures, the recombinant vectors, pET32/LIC-Omp34 plasmid was

created. Sequencing analysis showed that the gene of interest was authentic and kept in frame with the vector sequence. It was confirmed that the inserted gene as the Omp 34 kDa gene of *Brucella melitensis* strain 293 and was found to contain 927 bp. The gene encodes a deduced protein of 309 amino acids. Analysis of the nucleotide sequence of Omp 34 kDa gene of *Brucella melitensis* strain 293 revealed 100% homology to the porin of *Brucella melitensis* bv. 1 strain 16M and 99% to 98% homology to the *Brucella melitensis* porin (Omp2a) and (Omp2b) genes.

In developing the recombinant cell expressing the Omp 34 kDa, SDS-PAGE and Western immunoblotting analyses revealed that the expressed fusion protein of the pET32/LIC-Omp34 was approximately 51 kDa, which contained 17 kDa of tagged protein and remaining is 34 kDa of the Omp. The results conclusively demonstrated the successful expression of *Brucella melitensis* Omp 34 kDa gene as a fusion protein, which was tested in an *Escherichia coli* strain.

The next experiment involved *in vivo* efficacy of the recombinant *Escherichia coli* cells in stimulating humoral and cell-mediated immune responses in goats. During the course of study, both serum and blood from all groups; vaccinated and unvaccinated were collected to evaluate the antibody levels via enzyme-linked immunosorbent assay (ELISA) and cell mediated immunity response of $CD4^+$ and $CD8^+$ T cells via immunofluorescent assay. Overall, it was found that

goats immunized with recombinant cell or whole-cell followed by a booster dose on day 14, showed strong specific and significantly higher ($p<0.05$) IgG response when compared to the unvaccinated group throughout the entire 8-week study period. Significantly ($p<0.05$) high antibody levels was observed as early as week 1 post-vaccination and the titers was considerably increased after boosting. In contrast, the systemic cellular immune response, particularly the $CD4^+$ and $CD8^+$ T cells did not increase after the first and second exposures to the recombinant cells, but significant ($p<0.05$) responses cells were observed only at weeks 4 and 5. This was much inferior than the response following exposures to whole-cell that resulted in significant ($p<0.05$) increased as early as week 1 post-exposure. These results demonstrated that despite the high antibody level of IgG responses, vaccination with the recombinant *Escherichia coli* cells expressing the OMP34 kDa gene of *Brucella melitensis* strain 293 was capable of eliciting delayed systemic $CD4^+$ and $CD8^+$ T cells responses that lasted for a short period of time.

This study revealed that the Omp 34 kDa of *Brucella melitensis* strain 293 was immunogenic. Following preparation of recombinant cells expressing the gene encoding 34 kDa Omp, exposures to goats were capable to elicit high antibody levels. However, the systemic $CD4^+$ and $CD8^+$ T cells responses was delayed and lasted for a short period of time. Thus, further study is needed with the intention of boosting the cell-mediated immune responses.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains Veterinar

MENGEKSPRESI GEN 34-KILODALTON PROTEIN SELAPUT LUAR *Brucella melitensis* UNTUK PEMBANGUNAN VAKSIN REKOMBINAN

Oleh

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June 2011

Pengerusi : Profesor Mohd. Zamri Saad, DVM, PhD

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Brucellae merupakan bakteria Gram-negatif intrasel yang menyebabkan penyakit yang teruk pada manusia dan haiwan. Brucellosis masih endemik di banyak negara membangun, mempengaruhi kesihatan haiwan dan produktiviti menyebabkan kerugian ekonomi yang besar. *Brucella melitensis* menyebabkan keguguran pada biri-biri, kambing dan lembu, dan dianggap spesis *Brucella* yang paling berbahaya bagi manusia. Setakat ini, pemvaksinan diterima sebagai cara terbaik untuk mengawal penyakit brucellosis pada ruminan kecil, dengan tujuan menurunkan kadar penyakit tersebut ke tahap yang boleh diterima. Di antara vaksin yang sedia ada, *Brucella melitensis* Rev 1 strain halus yang dilemahkan sering diguna untuk mengawal jangkitan *Brucella melitensis* pada ruminan kecil dengan memberikan perlindungan heterologi terhadap jangkitan oleh *Brucella*

Ovis. Kini, ia dianggap sebagai vaksin terbaik untuk biri-biri dan kambing. Namun, vaksin Rev 1 mempunyai beberapa kelemahan, iaitu sisa kevirulenan yang mampu menyebabkan keguguran pada haiwan bunting, kemampuan untuk menghasilkan antibodi terhadap lipopolisakarida licin (S-LPS) yang mengganggu diagnosis pembezaan antara pemvaksinan dan terjangkit; tahan terhadap streptomisin, iaitu salah satu antibiotik pilihan untuk merawat brucellosis; patogenik bagi manusia; dan penggunaannya dilarang di negara bebas *Brucella melitensis*. Kelemahan-kelemahan ini menunjukkan perlunya vaksin yang lebih baik dihasilkan untuk pembenteras brucellosis.

Kajian ini dilakukan untuk menganalisis profil protein selaput luar (Omp) dan menentukan keantigenan *Brucella melitensis* isolat tempatan*,* diikuti dengan penghasilan sel rekombinan mengekspresi Omp terpilih dan seterusnya mengkaji gerakbalas keimunan selepas didedahkan kepada sel rekombinan tersebut. Omp dari *Brucella melitensis* isolat tempatan strain 152, 183, dan 293 yang diasing menggunakan sodium dodecil sulfat- gel elektroforesis poliakrilamide (SDS-PAGE) menghasillkan tiga jalur utama dan empat jalur kecil yang ditemui dalam semua isolat. Mereka adalah jalur utama 25, 34 dan 89 kDa dan jalur kecil 16, 30, 43 dan 70 kDa. Imunobloting menunjukkan keputusan konsisten jalur antigenik dikongsi semua isolat, iaitu jalur 16, 34 dan 70 kDa. Hanya 34 kDa merupakan jalur luar utama yang antigenik.

Siasatan pada peringkat molekul melibatkan pemencilan dan pengesanan gen yang mengkodkan Omp 34 kDa Omp dari *Brucella melitensis* strain 293. Analisis jujukan menunjukkan gen yang terlibat adalah tulen dan berada pada kedudukan yang betul di dalam jujukan vektor. Ia mengesahkan bahawa gen yang terlibat adalah gen 34 kDa Omp *Brucella melitensis* strain 293 dan ditemui mengandungi berat molekul 927 bp serta mengkodkan protein pada 309 asid amino. Analisis jujukan nukleotida gen Omp 34 kDa *Brucella melitensis* strain 293 mendedahkan 100% adalah persamaan dengan porin *Brucella melitensis* bv. 1 strain 16M dan 99% hingga 98% persamaan kepada gen porin *Brucella melitensis* (Omp2a) dan (Omp2b).

Dalam proses membangunkan rekombinan sel yang mengekspresi Omp 34 kDa, analisis SDS-PAGE dan Western imunoblotting mendedahkan bahawa protein lakuran yang diekspresikan dari pET32/LIC-Omp34 adalah kira-kira 51 kDa, yang terdiri daripada 17 kDa label protein dan 34 kDa Omp. Keputusan menunjukkan kejayaan dalam mengekspresi gen Omp 34 kDa *Brucella melitensis* sebagai protein lakuran dalam strain *Escherichia coli*.

Kajian seterusnya adalah untuk menentukan secara *in vivo,* keberkesanan sel rekombinan *Escherichia coli* dalam merangsang gerakbalas imun humoral dan sel oleh kambing. Sepanjang kajian ini dijalankan, serum dan darah dari semua kumpulan; divaksinasi dan tidak divaksinasi diambil untuk menentukan paras antibodi melalui assai imunoerap terangkai enzim (ELISA) dan gerakbalas sel

$CD4^+$ dan $CD8^+$ sel T melalui asei immunofluorescent. Secara keseluruhan, didapati bahawa kambing yang divaksinasi sama ada dengan sel rekombinan atau sel seluruh, diikuti dos penguat pada hari ke-14, menunjukkan paras antibodi IgG yang kuat dan meningkat secara signifikan ($P<0.05$) bila dibandingkan dengan kumpulan tidak divaksinasi. Secara nyata ($p <0.05$) paras antibodi tinggi dilihat seawal minggu pertama selepas pemvaksinan dan titer terus meningkat selepas pemberian dos penguat. Walau bagaimanapun, gerakbalas imun sistemik sel $CD4^+$ dan $CD8^+$ sel T tidak meningkat selepas dedahan pertama dan kedua pada sel rekombinan, tetapi gerakbalas signifikan ($p <0.05$) hanya berlaku pada minggu ke 4 dan 5. Keputusan ini menunjukkan bahawa walaupun gerakbalas antibodi IgG adalah tinggi, pemvaksinan dengan sel rekombinan *Escherichia coli* mengekspresi gen Omp34 kDa dari *Brucella melitensis* strain 293 hanya mampu untuk menghasilkan gerakbalas lewat oleh sel $CD4^+$ dan $CD8^+$ sel T untuk jangka masa yang pendek.

Sebagai kesimpulan, kajian ini menunjukkan bahawa Omp 34 kDa *Brucella melitensis* strain 293 adalah imunogenik. Pendedahan kepada sel rekombinan yang mengekspresi gen Omp 34 kDa dari *Brucella melitensis* mampu meningkatkan paras antibodi IgG yang tinggi. Akan tetapi, gerakbalas sistemik sel $CD4^+$ dan $CD8^+$ adalah lewat dan untuk masa yang singkat. Dengan demikian, kajian lanjut diperlukan dengan tujuan meningkatkan gerakbalas imun berperantara sel.

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I certify that an Examination Committee has met on 14 June 2011 to conduct the final examination of Yulianna Puspitasari on her Master of Veterinary Science thesis entitled "Expressing The Gene Encoding 34-Kilodalton Outer Membrane Protein of *Brucella melitensis* for Development of A Recombinant Vaccine" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommended that the candidate be awarded the relevant degree.

Members of the Examinations Committee were as follows:

The Thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Veterinary Science. The members of the Supervisory Committee were as follows:

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Date:

DECLARATION

I declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that is has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

_____
YULIANNA PUSPITASARI

Date: 14 June 2011

Dedication

The thesis is dedicated special for my lovely little family : pipi, ibram, nuno and also for my lovely papa, mama, bapak, ibu ... my luv always never end ....

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LIST OF ABBREVIATIONS

%	Percentage
α	Alpha
β	Beta
Γ	Gamma
Δ	Delta
°C	Degree celcius
µg	Microgram
µl	Microliter
µm	Micronmeter
µM	Micromolar
Amp^R	ampicillin resistance
APC	Antigen Presenting Cells
APS	Ammonium persulfate
BLAST	Basic local alignment search tool
Bp	Base pair
BSA	bovine serum albumin
CD	Cluster of Differentiation
Cfu	colony forming unit
CMI	Cell Mediated Immunity
CO_2	Carbon dioxide
CSF	Colony-Stimulating Factor

CTL's	Cytotoxic T lymphocytes
Cu	Copper
DC's	Dendritic Cells
DDT	Dithiothreitol
DMSO	Dimethylsulfoxide
DNA	deoxyribonucleic acid
Dntp	deoxynucleotide triphosphate
Ds	Double-stranded
EDTA	Ethylene-diamine-tetraacetic acid (disodium salt)
ELISA	enzyme linked immunosorbent assay
G	Gram
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
H_2O	Water
H_2S	Hydrogen Sulfide
Hsp's	Heat shock proteins
Htr	High-temperature-requirement
i.e.	in example
IFN	Interferon
IgG	immunoglobulin G
IL	Interleukin
In vitro	in an experimental situation outside the organism. Biological or chemical work done in the test tube (in vitro is Latin for "in glass") rather than in living systems.

In vivo	in a living cell or organism
IPTG	isopropyl-β-D-thiogalacosidase
Kb	kilobase pair
kDa	Kilodalton
LB	Luria-Bertani
LPS	Lipopolysaccharide
L	Liter
M	Molar
mA	Miliampere
mAB	monoclonal antibody
MCS	Multiple cloning site
$MgCl_2$	magnesium chloride
Mg	Milligram
MHC	Major Histo-compatibility Complex
Min	Minutes
Ml	Milliliter
mM	Milimolar
$MgCI_2$	magnesium chloride
mRNA	messenger ribonucleic acid
MW	molecular weight
Na_2HPO_4	di-sodium hydrogen phosphate
NaCl	Natrium chloride

NaH_2PO_4	Sodium di-hydrogen peroxide
NaOH	Sodium hydrogen peroxide
Ng	nanogram
NK	Natural Killer
Nm	Nanometer
OD	optical density
Omp	outer membrane protein
Ori	Origin
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pET32/LIC-Omp34	recombinant plasmid (pET-32/LIC+Omp 34 kDa gene of *Brucella melitensis*)
Ph	puissance hydrogen (Hydrogen-ion concentration)
Pmol	pico-mol
PVDF	polyvinyl difluoride
RBS	ribosome binding site
Rpm	rotation per minute
RT	Room temperature
S	Seconds
SDS	Sodium dodecyl sulphate
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	Superoxide Dismutase

Taq	*Thermus aquaticus* YT-1
TBE	Tris-boric EDTA
TBS	Tris-buffer saline
TE	Tris-EDTA buffer
TEMED	*N,N,N′,N′*-tetramethylethylene diamine
Th	T helper
TLR	Toll-like receptors
T_M	Melting temperature
TNF	Tumor Necrosis Factor
Tris-HCI	Tris (hydroxymethyl) aminomethane hydrochloride
U	Unit
UV	Ultra-violet
V	voltan/volt
v/v	Volume per volume
w/v	weight per volume
Zn	Zinc

Amino acid	Single/Three letter	Amino Acid Code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid	D	Asp
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Isoleucine	I	Ile
Leucine	L	Leu
Lycine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Valine	V	Val

CHAPTER 1

INTRODUCTION

Brucellosis is a zoonotic disease caused by several species of bacteria from the genus *Brucella*. Brucellosis in goats is caused by *Brucella melitensis*, and is responsible for considerable economical losses to the goat farmers by way of reproductive losses in the form of abortions and stillbirths (Gupta *et al*., 2007). *Brucella melitensis* infection is one of the most widespread and important zoonoses in the world. The disease is still widespread throughout most countries of the Mediterranean Basin, West Asia and some parts of Latin America (Benkirane, 2006).

The predominant symptom of an acute *Brucella melitensis* infection is reproductive failure with abortion or birth of weak offspring. Abortion occurs during the last two months of gestation (Cutler *et al*., 2005). In males the reproductive organs are affected and the bacteria may be shed with semen. Persistent infection of the udder is accompanied by intermittent discharge of the agent in milk. Inflammation of the mammary gland reduces milk production. Aborted foetuses may show increased amounts of bloody fluids in their body cavities and enlarged spleen and liver. Foetal membranes may be oedematous or necrotic.

Strategies for the prevention and control of brucellosis in small ruminants population depend on the prevailing epidemiological and socio-economic conditions (Minas, 2006). Eradication of brucellosis in small ruminants can be achieved mainly by depopulation of the infected flocks, but this strategy is costly and needs a good organization of farmers and veterinary service with simultaneous implementation of strict movement control measures so that the disease will not be reintroduced (Minas, 2006). Eradication by implementation of test and slaughter program cannot be universally applied since it is possible under certain conditions (Blasco, 2006). Thus, application of a control program based on vaccination to reduce the prevalence of the disease to an acceptable level is by implementing an appropriate control measure of brucellosis in small ruminant in many countries (Blasco, 2006).

The best classical vaccine available for controlling *Brucella melitensis* infection is the vaccine which consists of live attenuated *Brucella melitensis* REV-1, which rapidly enhanced the immunity of a flock (Minas, 2006). However, vaccination with the REV-1 has significant drawbacks, namely the development of strong antibody responses indistinguishable from those induced by natural infection with *Brucella melitensis* (Garin *et al*., 1998), induction of abortion when administered during pregnancy and is pathogenic to humans (Blasco and Diaz, 1993).

Another vaccine, a smooth-strain *Brucella melitensis* H38, prepared as formaldehyde-killed cells in mineral oil adjuvant, is capable to stimulated good

protection against abortion, but the vaccine produces positive serology and unacceptable local reactions (Renoux *et al*., 1964; Plommet *et al*., 1970; Meyer and Gibbons, 1978). *Brucella suis* strain S2 has been successfully used via oral vaccination to control brucellosis of small ruminants in field condition in China (Xin, 1986) and Libya (Mustafa and Abusowa, 1993). However, this vaccine appears to have no protective effect against *Brucella melitensis* infection in sheep (Verger et al., 1995). VTRM1 is a rough *Brucella melitensis* strain that does not interfere with classical serological test, but fails to confer adequate protection in goats (Elzer *et al*., 1998).

Therefore, identification of protective antigens is important for the development of future alternative vaccines, which would avoid the drawbacks of the live attenuated vaccines. Nucleic acid vaccination using naked DNA or recombinant plasmid both directly or through viral or bacterial vector is an interesting approach to boost the immune system. Thus, this approach could be used to develop a new generation of anti-brucellosis vaccines in the near future (Kurar and Spliter, 1997).

The outer membrane proteins (Omps) are among the suitable candidates for preparation of subunit vaccine against brucellosis. The major Omps of *Brucella* spp. have been extensively characterized as potential immunogenic and protective antigens (Cloeckaert *et al*., 2002). However, the Omps of local isolates of *Brucella melitensis* have not been analysed for their potentiality as a vaccine

candidate. For that reason, the potential of recombinant-based vaccine encoding outer membrane protein of local isolates *Brucella melitensis* will be evaluated in this study. Therefore, the objectives of this study were:

1. to analyze the Omp profile and determine the antigenicity of local isolates of *Brucella melitensis*.

2. to construct a recombinant vector containing a gene encoding the selected Omp of local isolate of *Brucella melitensis* and express the gene in *Escherichia coli*.

3. to study the immune responses of a killed recombinant *Escherichia coli* cell expressing the Omp of a local isolate of *Brucella melitensis*.

The hypothesis of this study were:

1. the Omp31 of local isolates of *Brucella melitensis* is the most antigenic.

2. the gene encoding the Omp31 of *Brucella melitensis* can be successfully cloned and expressed in *Eschericia coli*.

3. the killed recombinant *Eschericia coli* cell expressing the Omp31 of a local isolate of *Brucella melitensis* can stimulate immune response.

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