UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND EVALUATION OF RECOMBINANT OUTER MEMBRANE PROTEINS I-ELISA FOR DIAGNOSIS OF CAPRINE BRUCELLOSIS

IHSAN MUNEER AHMED

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By

IHSAN MUNEER AHMED

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

December 2013

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DEDICATION

To my loving mother and memory of my late father for their kindness and love.

To my wife, my daughters and my son; I greatly appreciate your support.

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia
in fulfillment of the requirements for the degree of Doctor of Philosophy

DEVELOPMENT AND EVALUATION OF RECOMBINANT OUTER MEMBRANE PROTEINS I-ELISA FOR DIAGNOSIS OF CAPRINE BRUCELLOSIS

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December 2013

Chairman: Associate Professor Siti Khairani Bejo, PhD

Faculty: Veterinary Medicine

Brucella melitensis is the main etiological agent of caprine brucellosis. The common serological tests for diagnosis of brucellosis include: Rose Bengal plate test (RBPT), complement fixation test (CFT) and enzyme linked immunosorbent assay (ELISA). These tests usually detect antibodies against smooth lipopolysaccharide (LPS). Therefore, they may give false positive serological reactions (FPSR) due to vaccination with *B. melitensis* Rev.1 vaccine strain or natural infection by a number of Gram negative bacteria, mainly *Yersinia enterocolitica* O:9. The insufficiency of the current serological tests to differentiate the goats with FPSR leads to erroneous decision to cull them and resulting in unnecessary loss of animals. The results of previous researches using single recombinant outer membrane protein (rOMP) of *Brucella,* such as OMP28 or OMP31 showed lack of sensitivity. Therefore, combination of more than one rOMP might improve the sensitivity of the diagnostic test. Accordingly, this study was

conducted to develop and evaluate in-house rOMPs I-ELISA based on combination of rOMP25, rOMP28 and rOMP31 to differentiate FPSR using mouse model and goats as the natural host of *B. melitensis*.

The whole cell protein profiles of six *B. melitensis* field isolates were characterized using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The results revealed overall similarities among these isolates. Therefore, only the goats isolates studied for their OMPs antigenicity by immunoblotting using rabbit serum raised against *B. melitensis* strain 0331 field isolate. The results confirmed the presence of OMP25, OMP28, and OMP31 as immunogenic protein candidates.

Accordingly, the *omp25*, *omp28* and *omp31* genes of *B. melitensis* strain 0331 field isolate were amplified by PCR and cloned using pET-32 Ek/LIC prokaryotic system. The nucleotide sequences of these three genes were successfully submitted to the GenBank. The expression of the *omp25*, *omp28*, and *omp31* genes were studied in *E. coli* BL21(DE3). The purified expressed recombinant fusion proteins were analyzed by Western immunoblotting and the results revealed that rOMP25, rOMP28 and rOMP31 fusion proteins were immunogenic and in properly folded form, suggesting their usefulness as antigenic candidates for serological diagnosis of brucellosis.

The rOMP25, rOMP28 and rOMP31 were combined and named as rOMPs and used to develop in-house rOMPs I-ELISA which was evaluated using serum samples obtained from three groups of BALB/C mice. Group 1 was infected with *B. melitensis* strain 0331

field isolate, while group 2 and 3 were vaccinated with *B. melitensis* Rev.1 vaccine strain, and infected with *Y. enterocolitica* O:9 respectively. Using RBPT, no significant differences were found in the immune response among the three groups. On the other hand, the in-house rOMPs I-ELISA was able to differentiate the group 1 from FPSR due to either vaccination by *B. melitensis* Rev. 1 vaccine strain (group 2) or infection by *Y. enterocolitica* O:9. (group3).

The in-house rOMPs I-ELISA was further evaluated using serum samples from four groups. The groups comprised: naturally infected goats with *B. melitensis*, goats free from *Brucella* infection, goats vaccinated with *B. melitensis* Rev. 1 vaccine strain and random field samples from goats with unknown *Brucella* status. All the sera were tested using the in-house rOMPs I-ELISA, RBPT, BRUCELISA-400SG and CFT. When samples from vaccinated goats were tested, RBPT, BRUCELISA-400SG and CFT categorized these samples as positive. This implies that the common serological tests were not able to differentiate truly infected animals from those vaccinated, while the in-house rOMPs I-ELISA developed was able to differentiate between the vaccinated and infected goats with 94.44% sensitivity and 84.62% specificity.

In conclusion, the newly developed in-house rOMPs I-ELISA was able to differentiate vaccinated goats as a cause of FPSR, which indicate that this test has the potential diagnostic abilities over the commonly used tests; hence it can support the diagnosis of caprine brucellosis and potentially save animals from being erroneously culled in testing and culling serosurveillance efforts.

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

PEMBANGUNAN DAN PENILAIAN I-ELISA REKOMBINAN MEMBRAN LUAR PROTEIN UNTUK DIAGNOSIS BRUSELOSIS PADA KAPRIN

Oleh

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Brucella melitensis adalah agen etiologi utama penyakit bruselosis pada kaprin. Ujian serologi sepunya untuk diagnosis bruselosis adalah termasuk: ujian plat Rose Bengal (RBPT), ujian pengikatan komplemen (CFT) dan assai imunoerap terangkai enzim (ELISA). Ujian tersebut diatas biasanya mengesan antibodi terhadap lipopolisakarida (LPS) licin. Oleh itu, ujian tersebut boleh memberi tindakbalas serologi positif palsu (FPSR) yang disebabkan oleh pemvaksinan dengan vaksin *B. melitensis* Rev.1 atau jangkitan semulajadi oleh beberapa bakteria Gram-negatif, terutamanya *Yersinia enterocolitica* O:9. Kekurangan ujian serologi yang dapat membezakan diantara kambing yang benar-benar dijangkiti dan FPSR menyebabkan keputusan yang salah dibuat dalam proses penakaian dan seterusnya mengakibatkan kerugian kerana menakai haiwan yang tidak berpenyakit. Keputusan kajian terdahulu yang menggunakan protein

rekombinan membran luar (rOMP) tunggal daripada *Brucella*, seperti OMP28 atau OMP31 menunjukkan sensitiviti yang kurang. Oleh sebab itu, gabungan lebih daripada satu rOMP mungkin dapat meningkatkan kepekaan ujian. Oleh itu, kajian ini dijalankan untuk membangunkan rOMPs I-ELISA berdasarkan gabungan rOMP25, rOMP28 dan rOMP31; dan seterusnya menilai keupayaannya untuk membezakan FPSR menggunakan model tikus dan kambing iaitu perumah semulajadi bagi *B. melitensis*

Pencirian profil protein seluruh sel meliputi enam isolat *B. melitensis* telah dilakukan menggunakan natrium sulfat dodecyl gel elektroforesis (SDS-PAGE). Pada keseluruhannya, keputusan menunjukkan persamaan dikalangan isolat tersebut. Oleh itu, hanya isolat dari kambing dipilih untuk dikaji keantigenan OMP melalui kaedah imunoblot menggunakan serum arnab yang dibangkitkan keimunannya terhadap isolat *B. melitensis* 0331. Keputusan imunoblot mengesahkan kehadiran OMP25, OMP28, dan OMP31 sebagai calon protein imunogenik.

Sehubungan dengan itu, gen *omp25*, *omp28* dan *omp31 B. melitensis* isolat 0331 telah dia mplifikasi oleh PCR dan diklon menggunakan sistem prokariot PET-32 Ek/LIC. Jujukan nukleotida bagi ketiga-tiga gen telah berjaya dihantar ke GenBank. Ekspresi gen-gen *omp25* itu, *omp28,* dan *omp31* seterusnya telah dikaji dalam *E. coli* BL21(DE3). Ekspresi gabungan protein rekombinan tulin telah dianalisis dengan menggunakan kaedah pemblotan Western dan keputusan menunjukkan bahawa gabungan protein rOMP25, rOMP28 dan rOMP31 adalah imunogenik serta dalam bentuk dilipat yang betul, menunjukkan kegunaannya sebagai calon antigen untuk kaedah serologi mengdiagnosis bruselosis.

rOMP25, rOMP28 dan rOMP31 telah digabungkan dan dinamakan sebagai rOMPs; dan seterusnya digunakan dalam kajian ini untuk membangunkan rOMPs I-ELISA. rOMPs I-ELISA yang dibangunkan telah dinilai menggunakan sampel serum yang diperolehi daripada tiga kumpulan tikus BALB/C. Kumpulan 1 telah diinokulasi dengan *B. melitensis* isolat 0331, manakala kumpulan 2 dan 3 masing-masing telah diinokulasi dengan vaksin *B. melitensis* Rev. 1 dan *Y. enterocolitica* O:9. Ujian RBPT menunjukkan tiada perbezaan yang signifikan didapati dalam tindakbalas imun antara ketiga-tiga kumpulan. Sebaliknya, rOMPs I-ELISA yang dibangunkan dalam kajian ini telah dapat membezakan kumpulan 1 daripada kumpulan 2 dan 3 dimana tindakbalas serologi positif palsu (FPSR) disebabkan oleh sama ada vaksinasi dengan *B. melitensis* Rev 1 vaksin atau jangkitan oleh *Y. enterocolitica* O: 9.

rOMPs I-ELISA yang dibangunkan dalam kajian ini telah dinilai keupayaannya menggunakan empat kumpulan sampel serum. Kumpulan serum tersebut adalah: kambing yang dijangkiti *B. melitensis* secara semula jadi, kambing bebas daripada jangkitan Brucella, kambing disuntik dengan vaksin *B. melitensis* Rev. 1 dan sampel rawak daripada kambing yang tidak diketahui statusnya. Semua serum telah diuji dengan rOMPs I-ELISA, RBPT, BRUCELISA-400SG and CFT. Apabila sampel serum dari kambing yang telah divaksinasi dengan vaksin *B. melitensis* Rev. 1 diuji menggunakan teknik RBPT, BRUCELISA-400SG dan CFT, didapati semua sampel adalah positif. Ini bermakna ketiga-tiga ujian serologi tersebut tidak dapat membezakan haiwan yang benar-benar dijangkiti *B. melitensis* daripada haiwan yang telah vaksinasi. Manakala rOMPs I-ELISA yang dibangunkan dalam kajian dapat membezakan antara

kambing yang divaksini dan kambing yang dijangkiti *B. melitensis* dengan 94.44% kepekaan dan 84,62% kekhususan.

Kesimpulannya, rOMPs I-ELISA yang baru dibangunkan dalam kajian ini dapat membezakan diantara kambing yang divaksini daripada yang dijangkiti oleh *B. melitensis*. Ini menunjukkan bahawa kaedah ujian serologi yang baru dibangunkan berkebolehan mengatasi masalah FPSR yang berlaku apabila menggunakan berbagai teknik ujian serologi yang sediada seperti RBPT, BRUCELISA-400SG dan CFT. Teknik ujian ini adalah amat berpotensi untuk digunakan sebagai teknik mengdiagnosis penyakit bruselosis pada kambing untuk menggantikan kaedah serologi yang sediada. Akhirnya, teknik serologi yang dibangunkan dalam kajian ini dapat menyelamatkan kambing yang tidak berpenyakit daripada ditakai.

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Ihsan Muneer Ahmed

December 2013

I certify that a Thesis Examination Committee has met on 6.12.2013 to conduct the final examination of **Ihsan Muneer Ahmed** on his Doctor of Philosophy thesis entitled **"Development and Evaluation of Recombinant Outer Membrane Proteins I-ELISA for Diagnosis of Caprine Brucellosis"** in accordance with the Universities and University College 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 degree of Doctor of Philosophy.

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DECLARATION

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

IHSAN MUNEER AHMED

Date: 6.12.2013

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%	percent
°C	degree Celsius
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometer
AGID	agar gel immunodiffusion
AP	alkaline phosphatase
APS	ammonium persulfate
AUC	area under the curve
BLAST	basic local alignment search tool
bp	base pair
BPAT	buffered antigen plate agglutination test
BSA	bovine serum Albumin
C-ELISA	competitive enzyme linked immunosorbent assay
CFT	complement fixation test
CFU	colony forming unit
CI	confidence interval
CMI	cell-mediated immunity
CO_2	carbon dioxide
Cu^{+2}	copper
dATP	deoxyadenosine triphosphate
dH_2O	distilled water
DDBJ	DNA data bank of Japan
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate

EDTA	ethylene-diamine-tetra acetic acid
Ef	efficiency
Ek/LIC	eukaryotic/ ligation independent cloning
ELISA	enzyme linked immunosorbent assay
EMBL-EBI	European Molecular Biology Laboratory-European Bioinformatics Institute
FAO	food and agriculture organization
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FN	false negative
FNSR	false negative serological reactions
FP	false positive
FPA	fluorescence polarization assay
FPSR	false positive serological reactions
g	gram
GST	glutathione-S-transferase
H_2O_2	hydrogen peroxide
H_2S	hydrogen sulfide
His.Tag	hexahistidine tag
HRP	horseradish peroxidase
I-ELISA	indirect enzyme linked immunosorbent assay
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IPTG	isopropyl β-D-1-thiogalactopyranoside
IS	insertion sequence
IU	international unit
JOVAC	Jordan Bio-Industries Center
K	kappa value

kb	kilobase pair
kDa	kilodalton
L	liter
LB	Luria-Bertani
LIC	ligation independent cloning
LPS	lipopolysaccharide
M	molar
mA	milliampere
MAbs	monoclonal antibodies
MBP	maltose-binding protein
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
MLST	multi-locus sequence analysis
MLVA	multiple-locus variable number of tandem repeats analysis
mm	millimeter
mM	millimolar
NCBI	national center for biotechnology information
ng	nanogram
NH	native hapten polysaccharides
nm	nanometer
NPV	negative predictive value
OD	optical density
OIE	world organization for animal health
OMP	outer membrane protein
OPS	O-polysaccharide
ORF	open reading frame

PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
pH	puissance hydrogen (hydrogen ion concentration)
PI	performance index
pmol	picomolar
PP	percentage of positivity
PPV	positive predictive value
RBPT	Rose Bengal plate test
RID	radial immunodiffusion
R-LPS	rough lipopolysaccharide
rRNA	ribosomal ribonucleic acid
ROC	receiver operating characteristics
rOMP	recombinant outer membrane protein
rOMP25	recombinant outer membrane protein 25
rOMP28	recombinant outer membrane protein 28
rOMP31	recombinant outer membrane protein 31
rpm	revolution per minute
RT	room temperature
SAT	serum agglutination test
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Se	sensitivity
sec	second
S-LPS	smooth lipopolysaccharide
SOC	super optimal broth with catabolite repression
Sp	specificity
spp.	species

Taq	*Themus aquaticaus*
TBE	tris-borate EDTA buffer
TBS	tris-buffered saline
TEMED	N,N,N,N-tetramethylene- diamine
Tm	melting temperature
TMB	tetramethylbenzidine
TN	true negative
TP	true positive
TPC	total plate count
TRX	thioredoxin
U	unit
USDA	United States Department of Agriculture
UV	ultraviolet
V	volt
VRI	Veterinary Research Institute
v/v	volume per volume
WHO	world health organization
w/v	weight per volume
× *g*	times gravity
Zn^{+2}	zinc

CHAPTER 1

INTRODUCTION

Brucellosis is one of the most important bacterial zoonoses worldwide. The disease has important economic and public health consequences (Franco *et al.*, 2007). *Brucella melitensis* is the main etiological agent of caprine brucellosis. It is also the main agent responsible for human brucellosis, known as Malta fever (Blasco and Molina-Flores, 2011). The presence of brucellosis in Malaysia was first confirmed since isolation of *B. abortus* from large ruminants in 1950. In small ruminants, brucellosis was first reported in sheep between 1987 and 1991 using serological methods, followed by isolation of *B. melitensis* biotype 2 from sheep in an outbreak in Johor, a southern state of Malaysia, in 1994 (Bahaman *et al.*, 2007). In goats, *B. melitensis* biovar 1 was isolated from serological positive animals in Kedah, a northern state of Malaysia (AL-Garadi *et al.*, 2011). In addition, a serological study on prevalence of brucellosis in goats in Negeri Sembilan, central region of Malaysia, in 2012 reported that 2.5% seropositivity among the tested goats (Sumayah, 2012). The serological prevalence and isolation of the causative agent suggested that brucellosis has potential major economic losses and public health impact to the country. At present, the disease appeared to have spread throughout the country (Sumayah, 2012).

The six classical species of the genus *Brucella* include *B. abortus, B. melitensis, B. suis B. neotomae,* which have smooth lipopolysaccharide (S-LPS), while *B. ovis* and *B. canis* have rough lipopolysaccharide (R-LPS) (Moreno *et al.*, 2002; Banai and Corbel, 2010).

The S-LPS is the major antigen of cell surface of all smooth *Brucella* species (Douglas and Palmer, 1988). Therefore, common serological tests like Rose Bengal plate test (RBPT), complement fixation test (CFT) and enzyme linked immunosorbent assay (ELISA) usually detect antibodies against S-LPS of *Brucella* (Poester *et al.*, 2010). As no differences have been found in antibody response, targeting S-LPS, between *B. melitensis* field strains and those from *B. melitensis* Rev.1 vaccine strain, this creates a major problem that vaccinated animals cannot be clearly differentiated from infected animals by the current serological tests and thus, giving rise to false positive serological reactions (FPSR) (OIE, 2009b; Nielsen and Yu, 2010). Another factor contributes to FPSR is the natural infection by a number of Gram negative bacteria, mainly, *Yersinia enterocolitica* O:9, *Salmonella* spp. and *Escherichia coli* O:157 which induce cross reacting antibodies (Weynants *et al.*, 1996; Velasco *et al.*, 1998). The insufficiency of the common serological test to differentiate the infected goats with FPSR leads to erroneous decision to cull these animals which resulted in unnecessary loss of animals, in addition to indirect costs of culling process especially in areas with low prevalence rates.

To overcome the disadvantages of the tests based on anti-LPS antibodies and reduce FPSR, an increasing interest was generated in the detection of antibodies to alternative antigens, mainly the outer membrane proteins (OMPs) and cytoplasmic proteins (Cloeckaert *et al.*, 1990; Baldi *et al.*, 1996). Interest in the *Brucella* OMPs stems initially from their potential as protective antigens (Cloeckaert *et al.*, 2002). The outer membrane of *Brucella* is mainly composed of major OMPs and among them, group 3 major OMPs

of *Brucella*, which consist of OMP25–27 kDa and OMP31–34 kDa, have been shown immunogenic reactivity using of monoclonal antibodies (Bowden *et al.*, 1995). Furthermore, this group of proteins and proteins below 20 kDa were only detected in sera of infected sheep, which indicates that these OMPs could be useful to differentiate *B. melitensis* infection from *B. melitensis* Rev. 1 vaccination in sheep and goats (Gupta *et al.*, 2007b). Although several single *omp* genes have been cloned and their expressed proteins were tested in immunoenzymatic assays for serodiagnosis of brucellosis in animals like OMP25 (Cloeckaert *et al.*, 1996c), OMP28 (Chaudhuri *et al.*, 2010) and OMP31 (Gupta *et al.*, 2007b), these proteins generally lack the sensitivity to detect the antibodies against the desired OMP. It seems likely that for maximum sensitivity, several of these proteins would have to be combined in a single immunoenzymatic test (Letesson *et al.*, 1997).

In situations where bacteriological examination and molecular methods are not practical such as when testing large numbers of samples, serological methods for the indirect diagnosis of brucellosis is more practical. Enzyme linked immunosorbent assay (ELISA) is considered an important serological procedure for the diagnosis of animal brucellosis (Garin-Bastuji *et al.*, 2006; OIE, 2009a, 2009b). Furthermore, ELISA provide excellent sensitivity and specificity whilst being robust, not complicated to run and have minimum requirements of equipments and reagents standardization (Nielsen *et al.*, 2005b).

Based on the above mentioned confirmations, our hypotheses were set based on, characterization of OMPs of *B. melitensis* field isolates could reveal the immunogenic properties of these proteins which may be useful as diagnostic antigen candidates for production of recombinant OMPs (rOMPs). Finally, the combination of these rOMPs might improve the sensitivity of the developed in-house rOMPs I-ELISA and reduce the number the FPSR goats in serological diagnosis of caprine brucellosis.

Therefore, objectives of this study were to:

1. Determine the antigenicity of OMP25, OMP28 and OMP31.
2. Express *omp25*, *omp28* and *omp31* genes using prokaryotic expression system.
3. Develop in-house rOMPs I-ELISA and evaluate its diagnostic performance in mouse model.
4. Evaluate the diagnostic performance of in-house rOMPs I-ELISA using goats serum samples.

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