UNIVERSITI PUTRA MALAYSIA

COMPARATIVE IMMUNOPATHOPHYSIOLOGICAL RESPONSES IN MICE FOLLOWING DIFFERENT ROUTE OF INOCULATION OF Brucella melitensis AND ITS LIPOPOLYSACCHARIDE

ABDINASIR YUSUF OSMAN

FPV 2016 25

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By

ABDINASIR YUSUF OSMAN

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

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DEDICATION

To the Almighty Allah who has been my help, sustainer, provider, guide, encouragement, and my all in all throughout the course of my studies.

To my late father ''May Allah blesses him with His supreme benevolence''.

To my caring mother and lovely wife who have shown me the unprecedented sacrifice to make sure we reach together the goal of the journey.

To my sister, brothers and all those who passed away in struggle for sovereignty of my fatherland.

To my patient and bleeding country, may Allah grant you peace.

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

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Chairman : Professor Abdul Aziz Saharee, PhD
Faculty : Veterinary Medicine

Brucella melitensis, which causes a small ruminant brucellosis in sheep and goats and Malta fever in humans, is believed to enter the host via ingestion, inhalation or direct contact of the organism with broken skin or mucous membranes. Among the consequences of the different routes of infection are septicaemia, increased permeability of blood vessels and presence of the organism in several organs. However, the oral and the respiratory tract may not be the only portal of entry and route of spread of *B. melitensis.* Circumstantial evidence had suggested the involvement of gastrointestinal, respiratory and reproductive tract in the pathogenesis of *B. melitensis* and its lipopolysaccharide in ruminants. Nevertheless, the pathogenesis and the immunopathophysiology of the disease following different route of infection have not been well documented since previous reports on the disease were limited to incidental observations. The response of gastrointestinal, respiratory, and reproductive tract following oral, intranasal, subcutaneous and intraperitoneal exposure to *B. melitensis* was studied and compared its severity with lipopolysaccharide (LPS) exposure. The cytokine, antibody pattern and sex related hormonal responses following the different route of inoculations to *B. melitensis* and its lipopolysaccharide in mice were also investigated.

The clinical signs observed in these studies include; inappetence, ocular discharge, and ruffled following the different route of exposure to *B. melitensis* and its lipopolysaccharide. Although the severity of the clinical sign varied over time, type of inoculum and route of inoculation, however, mean clinical score were significantly higher in oral and intraperitoneal exposed groups to *B. melitensis* followed by intranasal and subcutaneous groups, respectively. Clinical observations for intranasal and subcutaneous groups were limited mostly to mild and moderate involvement. In contrast to *B. melitensis* infected group, animals challenged with LPS showed mild clinical signs which seemed to be limited in the first 48 h post-

infection. Thereafter, normalization was observed in this group as they were not significantly different from those served as a control group. No significant differences were detected among the different sub groups of LPS infected indicating that the clinical presentation did not differ by route of exposure. Animals in control group did not develop any clinical signs throughout the experimental period.

The pathological alterations varied depending on the route of infection, days post-infection and the type of the organs recorded. Spleen, liver, kidney, lung and the reproductive organs that include uterus, ovary, testes, epididymis and seminal vesicle were the most commonly and severely affected organs with predominance in oral and intraperitoneally infected animals of *B. melitensis* group. These organs presented marked infiltration of inflammatory cells, degeneration, necrosis, haemorrhage and oedema. In intranasal and oral group of *B. melitensis*, lungs were the most affected organ than the other route of infection, with an abundance of fibrin admixed with cellular debris. Emphysema, oedema and marked infiltration of inflammatory cells were also recorded in lungs from 24 hours post-infection until the end point of the experiment. In contrast, histopathological changes of the various organs infected with LPS were almost similar presenting mild degrees of lesion involvement in all routes of infection with special reference in lungs and reticuloendothelial organs. Thus, indicating that LPS have preventive properties toward establishment of pathological lesions. Following the different routes of exposure, *B. melitensis* was isolated from the vital and reproductive organs along with intestinal segments of the mice that developed severe lesions scoring. Higher isolation and detection by PCR was noted predominantly in both reproductive tract and reticuloendothelial-rich organs of oral and intraperitoneal expose groups followed by intranasal and subcutaneous groups to *B. melitensis*, respectively.

Concurrently the cytokine and antibody immune response of mice following different routes of inoculation to *B. melitensis* and its lipopolysaccharide was also evaluated. Both *B. melitensis* and LPS elicited sustained and significantly higher serum Il-1β and IL-6 that has of minor relevance to the route of infection. However, the highest responses were noted in LPS group than *B. melitensis* infected group within the respective route of inoculation. Similarly, the LPS elicited sustained and significantly higher IgM and IgG levels than *B. melitensis* in all different routes of infection. Among the routes of infection, the subcutaneous group yielded highest titters of antibody response followed by intranasal and intraperitoneal groups, respectively. With the presence of severe histopathological evidence along with higher isolation of *B. melitensis* infected group in the reproductive tract, the experiment was conducted to evaluate the serum hormonal changes following different route of exposure to *B. melitensis* and its lipopolysaccharide. Both *B. melitensis* and LPS resulted in significant decrease in the circulating concentrations of serum progesterone, estradiol, and testosterone levels that has significant ($p<0.05$) difference when the effect is compared to those served as a control group.

This study showed that *B. melitensis* organisms were present in various segments and tissues of the gastrointestinal, respiratory, and reproductive tract following the different route of exposure. Therefore, it can be concluded that *B. melitensis*

infection can be transmitted via the gastrointestinal, respiratory and reproductive tract. Oral, intranasal and subcutaneous routes of administration of LPS elicited high serum cytokine and antibody immune response than *B. melitensis* infected group, although the responses of cytokines were variable. Thus, oral, intranasal and subcutaneous infections with 10^9 of live *B. melitensis* and its lipopolysaccharide were safer than the intraperitoneal route of inoculation. Both of these routes, in particular subcutaneous route, can be considered as potential alternative route for vaccine administration against *B. melitensis* infection in small ruminants. Similarly, it was concluded that the LPS stimulated significantly the innate and acquired immune system without significant systemic dysfunction, suggesting potentiality of the protective properties of this component as alternative vaccine for brucellosis infection.

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

PERBANDINGAN TINDAK BALAS IMMUNOPATOFISIOLOGIKAL DALAM TIKUS BERDASARKAN LALUAN INOKULASI *Brucella melitensis* DAN LIPOPOLYSACCHARIDE YANG BERBEZA

Oleh

ABDINASIR YUSUF OSMAN

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Brucella melitensis, yang menyebabkan brucellosis ruminan kecil dalam biri-biri dan kambing serta demam Malta pada manusia, dipercayai memasuki perumah melalui penghadaman, menghidu atau hubungan secara langsung organisma dengan luka pada kulit atau membran mukus. Di antra kesan jangkitan dari laluan yang berbeza adalah septisemia, kebolehtelapan saluran darah dan kehadiran organisma dalam beberapa organ-organ. Walau bagaimanapun, mulut dan saluran pernafasan adalah bukan satu-satunya portal kemasukan dan laluan penyebaran *B. melitensis*. Bukti dari keadaan semasa telah mencadangkan penglibatan saluran pencernaa, pernafasan dan saluran pembiakan dalam patogenesis *B. melitensis* dan lipopolysaccharide dalam ruminan. Walau bagaimanapun, patogenesis dan penyakit immunopatofisiologi penyakit ini berdasarkan laluan jangkitan yang berbeza masih belum didokumenkan atas sebab laporan-laporan penyakit yang terdahulu adalah terhad kepada pemerhatian yang berlaku secara tidak tiba-tiba. Tindak balas pendedahan saluran pencernaan, pernafasan, dan saluran pembiakan diikuti oral, intranasal, subkutaneus dan intraperitoneal kepada *B. melitensis* telah dikaji dan dibandingkan darjah keterukan dengan pendedahan lipopolysaccharide (LPS). Cytokine, corak antibodi dan tindakbalas hormon berkaitan seks berdasarkan laluan inokulasi *B. melitensis* dan lipopolysaccharide yang berbeza dalam mencit juga telah dikaji.

Tanda-tanda klinikal yang diperhatikan dalam kajian ini termasuk; kurang selera makan, penghasilan lelehan dari mata serta bulu haiwan yang tidak terurus berdasarkan pendedahan kepada *B. melitensis* dan lipopolysaccharide melalui saluran yang berbeza. Walaupun darjah keterukan tanda klinikal berubah dari masa ke masa selain jenis serta laluan inokulum, walau bagaimanapun, skor min klinikal adalah lebih tinggi dalam kumpulan oral dan intraperitoneal yang terdedah kepada *B. melitensis* diikuti dengan kumpulan intranasal dan subkutaneus. Pemerhatian klinikal

untuk intranasal dan kumpulan subkutaneus adalah terhad bagi kebanyakan penglibatan yang ringan dan sederhana. Sebaliknya, haiwan yang dijangkiti dengan LPS menunjukkan tanda-tanda klinikal yang sederhana yang mana ianya terhad dalam tempoh 48 jam selepas jangkitan. Sejurus itu, normalisasi diperhatikan berlaku dalam kumpulan ini kerana mereka tidak ketara berbeza daripada kumpulan kawalan. Tiada perbezaan yang signifikan telah dikesan di kalangan kumpulan sub berbeza dijangkiti LPS membuktikan kesan klinikal adalah tidak berbeza berdasarkan laluan pendedahan. Haiwan dalam kumpulan kawalan tidak menunjukkan apa-apa tanda-tanda klinikal sepanjang tempoh eksperimen.

Perubahan-perubahan patologi adalah berbeza-beza bergantung pada laluan jangkitan, bilangan hari selepas jangkitan dan jenis organ yang telah direkodkan. Limpa, hati, buah pinggang, paru-paru serta organ-organ pembiakan termasuklah rahim, ovari, testis, epididimis dan vesikel seminal adalah organ yang paling kerap terjejas teruk yang didominasi oleh haiwan dalam kumpulan yang telah dijangkit melalui laluan oral dan intraperitoneal. Organ-organ ini menunjukkan tingginya kehadiran sel radang, kemerosotan, nekrosis, pendarahan dan edema. Dalam kumpulan *B. melitensis* melalui intranasal dan oral, paru-paru adalah organ yang paling terjejas berbanding jangkitan melalui laluan yang lain, dengan kehadiran fibrin bercampur dengan serpihan selular. Emfisema, edema dan penyusupan sel-sel radang juga telah direkodkan dalam paru-paru dalam tempoh 24 jam selepas jangkitan sehingga titik akhir eksperimen. Sebaliknya, perubahan histopatologi pelbagai organ-organ dijangkiti LPS adalah hampir sama dengan penglibatan lesi secara sederhana dalam semua laluan jangkitan dengan rujukan khas dalam paru-paru dan organ-organ reticulo-endotelial. Oleh itu, ini menunjukkan bahawa LPS mempunyai ciri-ciri pencegahan kearah pengahasilan lesi patologi. Berikutan pendedahan laluan yang berbeza, *B. melitensis* telah diasingkan daripada organ-organ penting dan organ pembiakan bersama-sama dengan segmen usus mencit yang menunjukkan markah lesi yang teruk. Pengasingan yang lebih tinggi dan pengesanan oleh PCR telah dikenalpasti di peringkat awal dominasi bagi kedua-dua saluran reproduksi dan organ-organ yang kaya dengan reticuloendotelial dari kumpulan yang terdedah secara oral dan intraperitoneal diikuti oleh masing-masing kumpulan intranasal dan subkutaneus untuk *B. melitensis*.

Serentak dengan itu cytokine dan tindak balas imun antibodi dalam mencit berikutan laluan inokulasi *B. melitensis* dan lipopolysaccharide yang berbeza juga turut dinilai. Kedua-dua *B. melitensis* dan LPS turut mengalami penghasilan serum IL-1β dan IL-6 yang lebih tinggi yang mempunyai kesan yang sedikit kepada laluan jangkitan. Walau bagaimanapun, tindak balas tertinggi diperhatikan dalam kumpulan LPS berbanding kumpulan yang dijangkiti *B. melitensis* bagi aspek laluan inokulasi. Begitu juga, LPS didapati mengalami tahap IgM dan IgG jauh lebih tinggi berbanding *B. melitensis* dalam kesemua laluan jangkitan yang berbeza. Di antara laluan jangkitan tersbut, kumpulan subkutaneus menghasilkan tindak balas antibodi titer tertinggi diikuti masing-masing oleh kumpulan intranasal dan intraperitoneal. Dengan kehadiran bukti histopatologi yang teruk bersama-sama dengan pengasingan *B. melitensis* yang lebih tinggi bagi kumpulan dijangkiti dalam saluran pembiakan, eksperimen tersebut telah dijalankan untuk menilai perubahan hormon serum berdasarkan laluan pendedahan kepada *B. melitensis* dan lipopolysaccharide yang

berbeza. Kedua-dua *B. melitensis* dan LPS menyebabkan penurunan ketara dalam tahap kepekatan serum progesteron, estradiol, dan testosteron yang hanya mempunyai perbezaan yang ketara apabila kesan itu dibandingkan dengan kumpulan kawalan.

Kajian ini telah menunjukkan bahawa organisma *B. melitensis* hadir dalam pelbagai segmen dan tisu saluran pencernaan, pernafasan dan saluran pembiakan berikutan laluan pendedahan yang berbeza. Oleh itu, dapat disimpulkan bahawa jangkitan *B. melitensis* boleh berlaku melalui saluran pencernaan, pernafasan dan pembiakan. Laluan kemasukan LPS secara oral, intranasal dan laluan subkutaneus menghasilkan tinggi cytokine dalam serum dan tindak balas imun antibodi berbanding kumpulan yang dijangkiti dengan *B. melitensis,* walaupun tindak balas cytokines tersebut adalah berbeza-beza. Oleh itu, jangkitan *B. melitensis* dan lipopolysaccharide dengan dos yang besar secara oral, intranasal dan jangkitan subkutaneus adalah lebih selamat daripada laluan inokulasi intraperitoneal. Kedua-dua laluan ini boleh dianggap sebagai laluan alternatif yang berpotensi bagi peberian vaksin terhadap jangkitan *B. melitensis* ruminan kecil. Begitu juga, dapat disimpulkan bahawa LPS dapat merangsang sistem imun secara semula jadi dan imun yang diperlukan tanpa kegagalan fungsi sistemik yang ketara, menunjukkan potensi sebagai pelindung oleh komponen ini sebagai alternatif kepada pengahslan vaksin bagi jangkitan bruselosis.

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I certify that a Thesis Examination Committee has met on 2016 to conduct the final examination of Abdinasir Yusuf Osman on his PhD thesis entitled "Comparative immunopathophysiological responses in mice following different routes of inoculation to *Brucella melitensis* and its lipopolysaccharide" in Accordance with the Universities and University College Act1971 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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%	Percentage
˚C	Degree celcius
µg	Microgram
µL	Microlitre
µm	Micrometre
µM	Micrometre
1gM	Immununoglobulin M
1HC	Immuunohistochemistry
1L-β	Interleukin -1 β
ANOVA	Analysis of variances
APC	Antigen presenting cells
ASW	Predictive Analysis Software
B.melitensis	*Brucella melitensis*
BALB/c	Inbred strain of mouse
BHIB	Brain heart infusion broth
CFU	Colony forming unit
CPM	Count per minite
dH2O	distilled water
DNA	Deoxyribonucleic acid
DPI	Days post-infection
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
G	Group
G	Gram
GALT	Gut-associated lymphoid tissues
gDNA	Genomic deoxyribonucleic acid
GIT	Gastrointestinal tract
H	Hours
HE	Haematoxylin and Eosin
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase

IACUC	Animal Care and Use Committee
IFN-ϒ	Interferon gamma
IgA	Immunoglobulin A
IgG	Imununoglobulin G
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IN	Intranasal
IP	Intraperitoneal
L	Litre
LPS	Lipopolysaccharide
M	Moribund
$MgCL_2$	Magnesium Chloride
Min	Minutes
Ml	Millilitre
Mm	Millimetre
N	Number
NA	Not applicable
NaOH	Sodium hydroxide
NC	Negative Control
Ng	Nanogram
OD	Optical density
OIE	World Organization for Animal Health
OMP	Outer membrane protein
P1	Primer 1
P2	Primer 2
PASW	Predictive Analytics Software
PBS	Phosphate buffered saline
PBST	Tween20 phosphate buffered saline
PC	Positive Control
PCR	Polymerase chain reaction
Pg	Picogram
pH	Potential hydrogen/power of hydrogen (acidity or basicity)
PI	Post-infection
RIA	Radioimmunoassay

RNA	Ribonucleic Acid
Rpm	Revolution per minute
RT	Room temperature
S/c	Subcutaneous
Sec	Seconds
sIgA	Secretory iminunoglobulin A
SPSS	Statistical package for Social Sciences
T	Terminal
TAE	Tris-acetate-EDTA
TBE	Tris-boric acid-EDTA
TNFα	Tumour necrosis factor-alpha
TSB	Trypticase Soy Broth
UK	United Kingdom
UPM	University Putra Malaysia
USA	United States of America
USD	United States Dollar
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Brucella melitensis is one of the major zoonotic pathogens with significant economic implications as well as considerable human morbidity in many countries including Malaysia (Bamaiyi *et al.*, 2010; Seleem *et al.*, 2010). It is the main causative agent of small ruminant brucellosis (SRB) as it is also infectious to other species including cattle, buffalo and elk (Corbel, 2006; D áz, 2013). The disease remains endemic and neglected in many regions of the world, with predominance in the Mediterranean Basin, Middle East, Africa, Latin America and central Asia (Blasco & Molina-Flores, 2011; Lucero *et al.*, 2008; Thimm, 2013). The global burden of its incidence in human populations remains significantly at alarming rate (Pappas *et al.*, 2006).

The organism is facultative intracellular pathogen cocco-bacilli, non-spore-forming and non-capsulated with up to 3 biovars have been reported. These biovars differ biochemically by their pattern of metabolic activities (Halling *et al.*, 2005). The risk of brucellosis is presumed to be high in nomadic pastoral societies, laboratory workers or veterinarians where close and frequent contact between man and animals is part of the ecology.

The disease affects wild and domestic mammals with special predominance in small ruminants and cattle causing abortion and reduced fertility (Godfroid *et al.*, 2002; Gwida *et al.*, 2010; Megersa *et al.*, 2011). It is notifiable and neglected disease with serious economic repercussion on both humans and animals (Abernethy *et al.*, 2011; Ko & Splitter, 2003; Radostits *et al.*, 2007; Seleem *et al.*, 2010). It is mainly contracted through contact with placenta, foetus, foetal fluids and vaginal discharge from infected animals. In human, it is considered a food borne disease or a disease related to occupational exposures. The routes of infection for both humans and animals are similar of nature which include ingestion, inhalation, or through direct contact of the organism with a break in the skin (Corbel, 2006). Higher incidence of *B. melitensis* is associated with environmental and management factors which include moist, humid conditions, high animal population density, extensive free grazing system and poor husbandry practice (D áz, 2013). The initial symptoms of infected humans are fever, lethargy and night sweats. However, complication may set in as a result of chronic infection, which allows involvement of many organs and systems such as liver, spleen, kidney, and skeleton among others(Young *et al.*, 2014). In domestic animals, the disease is manifested as fertility-related issues. However, the most common symptom is usually abortion during the trimester often followed by retained placenta, weak offspring and metritis which may result in temporary infertility. Others include drop in milk production due to the infection of the udder. Rams experience orchitis and epididymitis. In addition, animals with polyarthritis have been observed in endemic flocks (Corbel, 2006; Radostits *et al.*, 2007).

Despite substantial attempts in the realm of the organism's characteristics, diagnosis and development of vaccines, the disease remains a major issue in animal industry (Bardenstein *et al.*, 2002; Corbel, 2006; Doganay & Aygen, 2003). The pathogenicity is complex and not always well understood. Understanding the pathogenicity and mechanism which *B. melitensis* interact with their hosts to produce clinical manifestation becomes a fundamental issue. Knowledge on immunopathophysiology of *Brucella* and its virulent factor is important to understanding the replication and survival of the bacteria.

The ability of the microbe to cause disease in a susceptible host, however, is determined by multiple virulence factors acting individually or together at different stages of infection (Neta *et al.*, 2010). In this regard, the presence of lipopolysaccharides (LPS) in the outer membrane protein of *B. melitensis* is believed to play a major role in diseases pathogenesis (Lapaque *et al.*, 2005). This unconventional non-endotoxic lipopolysaccharide confers resistance to anti-microbial attacks and modulates the host immune response (Lapaque *et al.*, 2005). The virulence factors are often involved in concealing the bacterial surface from the host's defense mechanisms. Their roles whether or not to directly mediate clinical manifestations of the disease is yet questionable. In the context of protection against *B. melitensis* infection, antibodies specific for the O-antigen of the lipopolysaccharide and production of proinflammatory cytokines are considered to be important for controlling *Brucella* infections (Macedo *et al.*, 2008; Neta *et al.*, 2010).

On the other hand, sex related hormones are essential for regulation of sex differentiation, reproduction, growth, metabolism and immune function (Mellon & Griffin, 2002; Murad *et al.*, 2010). A decrease in serum progesterone and estrogen levels is commonly associated with events leading to abortions in field conditions (Aisemberg *et al.*, 2013).

The clarifications, however, of the exact routes of transmission, sites of infection of *B. melitensis* and its LPS along with the impact of immunopathophysiological aspects in hosts can facilitate understanding of its biological features and control of brucellosis.

This study was, therefore, conducted to compare the establishment of clinical manifestation, the severity of pathological lesions, the role of innate and cellular immune response and the sex related hormonal alterations in mouse model following different route of inoculation of *B. melitensis* and its lipopolysaccharide (LPS).

1.1 R esearch hypotheses

1. Oral route of infection by *B. melitensis* produces comparable degree of injury and clinical manifestation of *B. melitensis* as intranasal, subcutaneous and/or intraperitoneal route of infection.
2. Pattern of bacterial distribution following experimental infection with *B. melitensis* in various organs and/or tissue is similar either orally, intranasally, subcutaneously and/or intraperitoneally infected mice.
3. All routes of administration of lipopolysaccharide (LPS) produce minimal tissue injuries and clinical manifestation compared to *B. melitensis.*
4. All routes of infection elicit comparable cytokine, hormonal changes and antibody immune responses following exposure to *B. melitensis* or its lipopolysaccharide (LPS).

1.2 Objectives of the study

1. To determine the clinical signs following experimental infection via different route inoculations of *B. melitensis* and its immunogens (LPS) in mouse model.
2. To determine the antibody levels (IgG and IgM) following assessment of different route inoculations of *B. melitensis* and its immunogens (LPS) in mouse model.
3. To measure the concentration of cytokines following experimental infection of mice with *B. melitensis* and its immunogens (LPS) via different route of inoculation.
4. To determine the concentration of reproductive hormones of both sexes following inoculation of animals with *B. melitensis* and its immunogens (LPS) via different route of exposure.
5. To evaluate the histopathological changes of the infected organs and tissues in mice following different route of exposure of *B. melitensis* and its immunogens (LPS).
6. To isolate and detect the *B. melitensis* by PCR from infected organs and tissues of mice challenged via different route of inoculation

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