UNIVERSITI PUTRA MALAYSIA

CLINICOPATHOLOGIC CHANGES ASSOCIATED WITH PASTEURELLA MULTOCODA B: 2 INFECTION AND ITS BACTERIAL LIPOPOLYSACCHARIDES AND OUTER MEMBRANE PROTEIN IN MICE AND CALVES

FAEZ FIRDAUS JESSE B. ABDULLAH @ JESSE A/L ARIASAMY

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BY
FAEZ FIRDAUS JESSE B. ABDULLAH @ JESSE A/L ARIASAMY

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

July 2011

DEDICATED WITH LOVE AND GRATITUDE
TO

WIFE: NUR FAEZA BT MOHD NOR
DADDY: ARIASAMY A/L ANTONY
MOTHER: SELVAKUMARI A/P USSIKATHAN
MOTHER IN LAW: NIK ZAHARAH
BROTHER NIXSON & SISTER NANCY

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

CLINICOPATHOLOGIC CHANGES ASSOCIATED WITH *PASTEURELLA MULTOCODA B: 2* INFECTION AND ITS BACTERIAL LIPOPOLYSACCHARIDES AND OUTER MEMBRANE PROTEIN IN MICE AND CALVES

By
FAEZ FIRDAUS JESSE B. ABDULLAH @ JESSE A/L ARIASAMY

July 2011

Chairman: Professor Hj Abdul Aziz Saharee, PhD

Faculty: Veterinary Medicine

Haemorrhagic septicaemia in cattle and buffaloes is an economically important livestock disease in Asia including Malaysia.

This study was conducted to investigate the host cell responses towards *Pasteurella multocida type B* and its immunogens which are the lipopolysaccharides (LPS) and outer membrane protein (OMP) in mice and cattle. Two hundred healthy male mice of eight to ten weeks of age and eight clinically healthy, non-pregnant and non-lactating Brangus cross calves weighing 150 ± 50 kg were used in this study. Throughout the experiments, three types of inocula were used that consisted of live wild-type *P. multocida* B:2, the lipopolysaccharide (LPS) of *P. multocida* B:2 and the outer membrane proteins (OMP) of *P. multocida* B:2 . The two hundred mice were divided into four equal groups of 50 mice each and the calves were divided into 4 groups of 2 cows in each group. The control group 1 (mice and calves) were inoculated with sterile

phosphate buffered saline (PBS) whereas group 2 were inoculated with live wild-type *P. multocida* and group 3 were inoculated with LPS broth extract. Animals (mice and calves) in group 4 was inoculated with OMP broth extract. All animals were observed for 48 hours (h) for clinical signs, changes in behavior and mortality pattern, including the time of death. Clinical scoring was done and the data was analysed using statistics. Blood samples were collected into plain, EDTA and sodium citrate containing tubes for blood and biochemistry analysis and acute phase protein analyses. Moribund animals were euthanized while the surviving animals were killed after 48 h. Post-mortem examination of gross lesions was conducted and lung, liver and heart samples were fixed for histopathology and cellular changes were scored and analysed using statistics. Mice and calves in all treated groups showed significant ($p<0.05$) changes in clinical signs compared to control group 1 with group 2 showed most severe clinical response. Mice and calves in all treated groups showed significant ($p<0.05$) increased in positive acute phase protein concentrations. Mice and calves in all treated groups showed significant ($p<0.05$) changes in blood and biochemistry parameters with differences in severity of changes. Mice and calves in all treated groups showed significant ($p<0.05$) cellular changes in the lung, liver and heart with different degree of severity of lesions.

Therefore, from this study, it can be concluded that the mice and calves in the positive control group and LPS immunogen groups showed similar clinical responses. For OMP immunogen group, there were differences in the severity of clinical responses between mice and calves, where in mice severe clinical responses were exhibited. Mice and calves were able to show significant increased in positive APPs. Hp and SAA were

sensitive APPs for both mice and calves for *P. multocida type B* infection and its immunogens. Mice and calves exhibited blood and biochemistry changes towards the immunogens. The cellular changes except for the prominence of kupffer cells in the three groups were similar in both mice and calves.

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

PERUBAHAN KLINIKOPATOLOGI BERKAITAN *PASTEURELLA MULTOCIDA B:2* DAN LIPOPOLISAKARIDA DAN PROTIN LUAR MEMBRAN BAKTERIA INI DALAM TIKUS DAN ANAK LEMBU

Oleh

Faez Firdaus Jesse b. Abdullah @ Jesse a/l Ariasamy

Julai 2011

Pengerusi: Profesor Hj Abdul Aziz Saharee, PhD

Fakulti: Perubatan Veterinar

Penyakit hawar berdarah pada lembu dan kerbau adalah penyakit berkepentingan ekonomi d Asia, termasuk Malaysia.

Kajian ini dibuat untuk menyiasat tindakbalas sel terhadap *Pasteurella multocida jenis B* dan imunogennya iaitu lipopolisakarida' (LPS) dan protin membrane luar (OMP) dalam tikus dan lembu. Sebanyak 200 ekor tikus jantan berumur di antara lapan hingga sepuluh minggu dan lapan anak lembu kacukan Brangus yang sihat secara klinikal dan yang tidak bunting dan tidak mengeluarkan susu serta mempunyai berat diantara 150 ± 50 kg telah digunakan dalam kajian ini. Sepanjang kajian, tiga jenis inokula telah digunakan yang terdiri daripada *P.multocida B:2* liar, LPS daripada *P.multocida B:2* liar dan OMP daripada *P.multocida B:2 liar*. Dua ratus ekor tikus telah dibahagikan sama rata kepada empat kumpulan dimana setiap kumpulan terdiri daripada lima puluh

ekor tikus manakala lembu pula telah dibahagikan kepada empat kumpulan dimana setiap kumpulan terdiri daripada dua ekor lembu. Bagi kumpulan kawalan 1 (tikus dan anak lembu) telah disuntik dengan 'phosphate buffered saline' (PBS), manakala kumpulan 2 telah diberikan *P.multocida B:2 liar* serta kumpulan 3 pula telah diinokulasi dengan larutan ekstrak "broth" LPS. *Haiwan (tikus dan anak lembu)dalam* kumpulan 4 pula telah diinokulasi dengan larutan OMP. Semua haiwan diperhatikan selama 48 jam bagi merekodkan tanda-tanda klinikal, perubahan sifat dan cara kematian berlaku termasuk waktu kematian. Skor tanda klinikal telah dilaksanakan dan data dianalisis menggunakan statistik. Sampel darah telah diambil menggunakan tiub darah jenis biasa, tiub yang mengandungi 'EDTA' dan 'Sodium citrate' bagi analisis darah dan biokimia dan juga analisis 'acute phase protein'. Haiwan yang menunjukkan tanda sakit yang kritikal dan haiwan yang hidup selepas 48 jam kajian akan dikorbankan. Pemeriksaan bedah siasat untuk memerhatikan lesi matakasar dan sampel paru-paru, jantung dan hati diawet untuk histopatologi dan pertukaran sel diskor dan dianalisis menggunakan statistik.

Tikus dan lembu daripada semua kumpulan kajian menunjukkan perubahan bermakna ($p<0.05$) dalam tanda-tanda klinikal berbanding dengan kumpulan kawalan 1 dengan kumpulan 2 menunjukan tanda klinikal paling teruk. Tikus dan lembu daripada semua kumpulan kajian menunjukan perubahan bermakna ($p<0.05$) dalam peningkatan kepekatan 'positive acute phase protein' dan juga perubahan bermakna ($p<0.05$) dalam analisis darah dan biokimia dengan kadar perubahan yang berbeza. Tikus dan lembu daripada semua kumpulan kajian menunjukan perubahan bermakna ($p<0.05$) dalam

perubahan sel paru-paru, jantung dan hati yang mempunyai tahap keterukan lesi yang berbeza.

Sebagai kesimpulan, tikus dan lembu dalam kumpulan kawalan positif dan LPS menunjukan tanda klinikal yang serupa manakala kumpulan OMP tikus dan lembu menunjukkan perbezaan dimana tikus mempamerkan tanda klinikal lebih teruk. Tikus dan lembu dapat menunjukkan peningkatan bermakna dalam positif APPs dimana protin Hp dan SAA adalah sensitif bagi kedua-dua tikus dan lembu untuk jangkitan *P.multocida B:2 liar* dan imunogennya. Tikus dan lembu menunjukkan perubahan darah dan biokimia terhadap imunogen. Perubahan sel kecuali kehadiran sel kupffer untuk semua kumpulan kajian tikus dan lembu adalah sama.

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I certify that an Examination Committee has met on ---------- to conduct the final examination of Faez Firdaus Jesse b.Abdullah @ Jesse a/l Ariasamy on his Doctor of Philosophy thesis entitled "Clinicopathologic changes associated with *Pasteurella multocida* B:2 infection, its bacterial lipopolysaccharide and outer membrane protein in mice and calves" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the examination Committee are as follows:

Members of the Examination Committee were as follows:

Dato' Abdul Rani Bahaman,PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia

Jasni Bin Sabri,PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Siti Khairini Bejo,PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Peter David Eckersall,PhD
Professor
Faculty of Veterinary Medicine
University of Glasgow,Scotland
United Kingdom
(External Examiner)

_____

HASANAH MOHD GHAZALI, PhD
Professor and Dean
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Abdul Aziz bin Saharee, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Rasedee bin Abdullah, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Mohd Zamri bin Saad, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Zunita binti Zakaria, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

_____
HASANAH MOHD GHAZALI, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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 other institutions.

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FAEZ FIRDAUS JESSE B. ABDULLAH @ JESSE A/L ARIASAMY
Date: 8 July 2011

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celcius
A/G ratio	Albumin Globulin Ratio
Ag	Antigen
cfu	Colony Forming Unit
ELISA	Indirect enzyme linked immunosorbent assay
LPS	Lipopolysaccharides
OIE	Office International des Epizooties
OMP	Outer Membrane Protein
P.multocida	*Pasteurella multocida*
SD	Standard deviation
S/C	Subcutaneous
IM	Intramuscularly
HS	Haemorrhagic Septicaemia
UPM	Universiti Putra Malaysia
VRI	Veterinary Research Institute
FAO	Food and Agriculture Organisation
I/P	Intra Peritoneal
I/V	Intravascular
Ca	Calcium
K	Pottasium
Cl	Chloride
Ca	Calcium

PT	Prothrombin Time
APTT	Activated Partial Thrombin Time
PP	Plasma Protein
Thromb	Thrombocytes
BO	Basophils
NO	Neutrophils
MO	Monocytes
LO	Lymphocytes
EO	Eosinophils
Seg NO	Segmented Neutrophils
Band NO	Band Neutrophils
Leuc	Leucocytes
MCHC	Mean Corpuscle Hematocrit cell
MCV	Mean Cell Volume
PCV	Packed Cell Volume
Hb	Haemoglobin
RBC	Red Blood Cell / erythrocytes
ALT	Alanine Transferase
AP	Alkaline Phosphatase
GGT	γ glutamil transferase
ALP	Aspartate Aminotranferase
CK	Creatinine Kinase
SAA	Serum Amyloid A

Hp	Haptoglobin
Fb	Fibrinogen
AGP	Alpha acid glycoprotein

CHAPTER 1

INTRODUCTION

The ruminant industry in Malaysia is gradually changing from subsistence to intensive operations (Jamaluddin, 1992). It comprises of large ruminants, mainly cattle and buffaloes and small ruminants, particularly goats and sheep. The ruminant industry plays an important role in the food industry through the production of milk, meat and the by- products such as hide for the leather industry. In Malaysia, the large ruminant sector is threatened by fatal, infectious disease outbreaks, which cause huge losses to farmers and the country. This acute fatal disease is known as haemorrhagic septicaemia (HS) caused by a Gram negative bacterium called *Pasteurella multocida* 6:B or B:2.

Pasteurella multocida is of considerable economic importance in the livestock industry (Collins, 1977). Infections by *Pasteurella multocida* have been reported in mammals and fowls (Soltys, 1979). It is an important primary or secondary animal pathogen for over a century and is becoming important as human pathogen (Biberstein, 1979) leading to a disease entity termed as pasteurellosis. Nevertheless, Pasteurellae have been shown to be a common microflora of the upper respiratory tract in normal animals (Campbell, 1983). The organisms usually act as secondary invaders in animals with concurrent diseases or suffering from debilitating stressful conditions (Benirschke *et al.,* 1978).

The serotype 6:B has been recovered from HS-affected animals in Southern Europe, Central and South America, the Middle East and Asia, including Malaysia. The infection causes substantial morbidity and mortality in cattle, buffaloes, sheep and goats (Bain *et al.,* 1982). In Malaysia, the stressful condition is during the raining season where most outbreaks occurred (Saharee *et al.,* 1993).

Haemorrhagic septicaemia in Malaysia is generally controlled by the use of killed whole cell vaccines. These vaccines have certain limitations, such as shorter duration of immunity and swelling at the site of inoculation. Moreover, outbreaks of HS have been reported to occur despite vaccinations (Chandrasekaran *et al.,* 1994b). Previous studies have suggested that capsular antigens, lipopolysaccharide (LPS) or LPS-protein complex and the outer membrane proteins (OMPs), including the iron-regulated OMPs as effective immunogens for serogroups B and E (Carter and De Alwis, 1989). Studies on immunization in mice with LPS of *P. multocida (6:B)* has shown that the protection observed was associated with LPS-associated proteins (Muniandy *et al.,* 1998). However, knowledge of host responses towards the outer membrane protein and lipopolysaccharide (LPS) of *P. multocida* in HS is still lacking. There is no documentation of complete blood count, blood biochemistry profiles, acute phase protein response and clinical responses during the infection by *P. multocida* B:2. There is also no report on the pathological changes in the host inoculated with the immunogens from *P. multocida.* This study is designed with the purpose of better understanding of the pathophysiological changes that can take place in the host using mice and cattle models. This will assist in the improvement of the vaccines and the

vaccination methods that we have currently employed to control this important disease in Malaysia.

The objectives of this study were as follow:

1. To observe and compare the clinical signs shown by mice and cattle following introduction of live *P.multocida B:2* and inoculation with the bacterial OMP and LPS.

2. To observe and compare the changes in the blood parameters of mice and cattle following infection by live *P. multocida B:2* and inoculation with the bacterial OMP and LPS.

3. To observe and compare the acute phase protein responses in mice and cattle following experimental infection by *P. multocida B:2* and inoculation with the bacterial OMP and LPS.

4. To observe and compare the cellular changes associated with infection by *P. multocida B:2* and inoculation with the bacterial OMP and LPS.

Therefore, the hypotheses were:

1. Infection by *P. multocida* B:2 will lead to most severe clinical signs compared to inoculation of LPS and OMP of *P. multocida B:2.*

2. There will be changes in the blood parameters of the host infected by live *P. multocida B:2* and inoculation with the bacterial OMP and LPS.

3. Both mice and cattle infected with *P. multocida B:2* and inoculated the bacterial OMP and LPS will show acute phase protein responses. However, it is hypothesized that acute phase protein responses by both mice and cattle will show no differences following experimental infection by *P.multocida B:2* and inoculation with the bacterial OMP and LPS.

4. There will be cellular changes in both mice and cattle when infected with *P. multocida B:2* and inoculated with the LPS and OMP of *P. multocida B:2.*

Thus, this study should provide a better understanding on the characteristics of *P. multocida* infection and the pathogenesis of pasteurellosis in the host.

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