UNIVERSITI PUTRA MALAYSIA

BUFFALOES' CLINICO-PATHOLOGICAL RESPONSES TO Pasteurella multocida TYPE B:2 AND THEIR IMMUNOGENS LIPOPOLYSACCHARIDE AND OUTER MEMBRANE PROTEIN

ERIC LIM TEIK CHUNG

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By

ERIC LIM TEIK CHUNG

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

October 2016

DEDICATION

My father (Lim Khong Chiu)
My mother (Ng Yoke Oi)
My wife (Lim Ai Phing)
My siblings (Felicia, Alvin and Andy)
And my friends

For their care, love, great source of motivation, inspiration, encouragement and endless support.

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

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Chairman: Associate Professor Faez Firdaus Jesse Abdullah, PhD
Faculty : Veterinary Medicine

Sudden death is usually the main finding in field during haemorrhagic septicaemia (HS) outbreaks among livestock in Malaysia. HS is an acute fatal disease, caused by particular serotypes of *Pasteurella multocida*. Epidemic haemorrhagic septicaemia in Asian countries including Malaysia is caused by P. *multocida* serotype B:2. This organism causes acute, highly fatal septicaemia with high morbidity and mortality in cattle and more susceptible in buffaloes. P. *multocida* is a gram negative, short, ovoid, bipolar staining coccoid forms. It is an extracellular parasite, and immunity is primarily humoral. In most cases, the clinical findings are either acute or peracute, resulting death within 8 to 24 hours after onset. The two immunogens of wild-type *Pasteurella multocida* that have the virulence factors are the outer membrane protein (OMP) and lipopolysaccharide (LPS). Little is known about the effect of endotoxin of P. *multocida* type B:2 and its immunogens LPS and OMP towards host cell responses in buffaloes. Thus, this study was designed to investigate the clinico-pathology, haemato-biochemistry changes, the acute phase responses (APP), antibody titres and cytokine concentrations in buffaloes infected by P. *multocida* type B:2 and its immunogens.

A total of twenty one buffalo heifers were divided equally into 7 treatment groups. Group 1 was inoculated orally with 10 mL of phosphate buffer saline (PBS) as negative control; Groups 2 and 3 were infected with 10 mL of 10^{12} colony forming unit (cfu) P. *multocida* type B:2 subcutaneously and orally respectively; Groups 4 and 5 were inoculated with 10 mL of LPS broth intravenously and orally respectively; and OMP broth were inoculated subcutaneously and orally into Groups 6 and 7 buffaloes respectively. During the post infection period, all the buffaloes were observed for clinical signs and clinical response for 21 days. Blood samples were also collected throughout the 21 days for determination of blood and biochemistry changes, concentration of proinflammatory cytokines, APP and antibody titres. At the end of the study, buffaloes that exhibited typical HS signs and buffaloes that survived throughout the 21 days study period were euthanized for post mortem and histopathological examination.

All buffaloes from Groups 1, 3, 4, 5, and 7 were able to survive throughout the stipulated experimental period of 21 days. Group 2 and 6 buffaloes were only able to survive for 12 hours and 3 days respectively. Group 2 buffaloes showed severe HS clinical responses and were only able to survive for 12 hours post infection. The blood and biochemistry results showed erythrocytosis, leukopaenia, neutropaenia and lymphopaenia. All vital organs, gastrointestinal and immune organs were severely affected with severe histopathology lesions. However, Group 3 buffaloes were able to survive throughout the experiment for 21 days despite showing mild clinical responses that only lasted for 4 days. The blood and biochemistry results showed leukocytosis for the first 5 days. Only the lung and liver organs were moderately affected with moderate histopathology lesions. On the other hand, Group 4 buffaloes demonstrated mild clinical responses and survived throughout the stipulated time of 21 days. All buffaloes had leukocytosis, lymphocytosis and monocytosis throughout 21 days experiment. All vital organs, gastrointestinal and immune organs were moderately affected with mild histopathology lesions. Similarly, Group 5 buffaloes demonstrated very mild clinical responses and able to survive for 21 days. Leukocytosis, monocytosis and eosinophilia were observed in this buffalo group. Only the lung and liver had mild gross lesions. Besides, mild histopathology findings were observed in all organs. Similar to Group 2, Group 6 buffaloes also exhibited severe HS clinical responses and were only able to survive for 72 hours post infection. The blood and biochemistry results showed monocytosis and elevated gamma glutamyl transferase (GGT) during the first 72 hours of the experiment prior euthanasia. All vital organs, gastrointestinal and immune organs were severely affected with moderate histopathology changes. In contrast, there were no significant clinical responses in Group 7 buffaloes. All buffaloes only had monocytosis throughout the 21 days of experiment. Gross lesions were only observed in the lung and liver of Group 3 buffaloes with moderate histopathological lesions. Buffaloes in all treatment groups of P. *multocida* type B:2 and its immunogens LPS and OMP showed significant increase in haptoglobin (Hp) and serum amyloid A (SAA) concentrations throughout the study period. There were significant differences ($p<0.05$) in all treatment groups compared to control group of buffaloes. In addition, buffaloes in all treatment groups showed significant increase in IgM, IgG and IgA concentrations throughout the study period. There were significant different ($p<0.05$) in all treatment groups compared to control group. Similarly, all treatment groups showed significant increase ($p<0.05$) in IL-2 and IL-6 concentration; but no significant difference ($p>0.05$) in IL-12 concentration observed compared to control group.

Therefore, from the present study, it can be concluded that buffaloes infected with P. *multocida* type B:2 and its immunogens LPS and OMP demonstrated different host cell responses. Information on this will play a significant role in understanding the host cell response during HS outbreaks. Buffaloes inoculated orally with OMP demonstrated as a promising candidate to be developed into oral vaccine based on the clinical response, haemato-biochemistry, gross lesion, histopathology finding, and most importantly antibody level. With this, alternative route for HS where oral route of vaccination can be considered compared to intramuscular route in future. Besides that, the development of biomarkers using cytokines in future will be able to control the outbreak of HS which causes major losses with great economic impact to the country.

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

TINDAK BALAS SEL BADAN KERBAU TERHADAP *Pasteurella multocida* B:2 DAN IMUNOGEN LIPOPOLISAKARIDA DAN MEMBRAN PROTIN LUAR

Oleh

ERIC LIM TEIK CHUNG

Oktober 2016

Pengerusi : Professor Madya Faez Firdaus Jesse Abdullah, PhD
Fakulti : Perubatan Veterinar

Kematian mengejut merupakan penemuan pertama pada haiwan semasa penyakit hawar berdarah di Malaysia. Penyakit ini disebabkan oleh serotype bakteria tertentu P. *multocida* yang mengakibatkan kematian dan septisemia dengan kadar jangkitan dan kematian yang sangat tinggi pada lembu dan terutamanya kerbau. Penyakit hawar berdarah di negara Asia termasuklah Malaysia disebabkan oleh P. *multocida* jenis B:2. P. *multocida* adalah bakteria gram negatif, pendek, bujur telur, bipolar apabila diwarnai. Ia adalah parasit luar sel dan imuniti terutamanya humoral. Dalam kebanyakan kes, tanda-tanda klinikal adalah sangat awal dan boleh menyebabkan kematian dalam masa 8 hingga 24 jam selepas dijangkiti. Membran protin luar dan lipopolisakarida merupakan imunogen terdapat pada bakteria P. *multocida* yang mempunyai faktor merbahaya. Tidak banyak yang diketahui tentang kesan endotoksin daripada P. *multocida* jenis B:2 dan imunogennya lipopolisakarida dan membran protin luar kepada sel badan kerbau. Oleh itu, kajian ini telah direka untuk menyiasat perubahan klinikal, darah, bedah siasat, histopatologi, kepekatan akut protein fasa, antibodi dan sitokin dalam kerbau selepas dijangkiti oleh P. *multocida* jenis B:2, lipopolisakarida dan membran protin luar.

21 ekor kerbau dibahagikan sama rata kepada 7 kumpulan rawatan. Kumpulan 1 disuntik dengan 10 mL air suling sebagai kawalan negatif; Kumpulan 2 dan 3 dijangkiti dengan 10 mL 10^{12} P. *multocida* jenis B:2 masing-masing secara injeksi subkutaneus dan oral; Kumpulan 4 dan 5 disuntik dengan 10ml lipopolysaccharide masing-masing secara intravena dan oral; dan 10 mL protein membran luar disuntik masing-masing secara subkutaneus dan oral untuk kerbau kumpulan 6 dan 7. Dalam tempoh jangkitan, semua kerbau diperhatikan tanda-tanda klinikal selama 21 hari. Sampel darah juga diambil sepanjang 21 hari bagi analisis darah dan untuk menentukan kepekatan akut protein fasa, antibodi dan sitokin. Pada akhir kajian ini, kerbau yang masih hidup selepas 21 hari disembelih dimana sampel organ pernafasan, jantung, hati, ginjal, organ imunisasi dan organ pencernaan diambil untuk pemeriksaan histopatologi.

Semua kerbau dari Kumpulan 1, 3, 4, 5, dan 7 berjaya hidup sepanjang tempoh 21 hari eksperimen. Kerbau Kumpulan 2 dan 6 masing-masing hanya mampu bertahan selama 12 jam dan 3 hari. Kerbau Kumpulan 2 menunjukkan respon penyakit hawar berdarah

dan hanya mampu hidup selama 12 jam selepas jangkitan. Keputusan darah dan biokimia menunjukkan peningkatan eritrosit dan penurunan sel darah putih. Semua organ yang diambil terjejas teruk dengan perubahan histopatologi yang teruk. Walau bagaimanapun, kerbau daripada Kumpulan 3 dapat bertahan sepanjang tempoh kajian iaitu selama 21 hari walaupun menunjukkan sedikit tanda klinikal untuk 4 hari yang pertama. Keputusan darah dan biokimia menunjukkan peningkatan sel darah putih bagi 5 hari yand pertama. Hanya organ paru-paru dan organ hati terjejas dengan perubahan histopatologi yang sederhana. Sebaliknya, kerbau daripada Kumpulan 4 menunjukkan sedikit tanda klinikal dan berjaya hidup sepanjang masa yang ditetapkan iaitu 21 hari. Semua kerbau menunjukkan peningkatan sel darah putih, limfosit dan monosit sepanjang 21 hari eksperimen. Semua organ-organ penting, pencernaan dan organ-organ imun terjejas dengan sedikit perubahan histopatologi. Begitu juga untuk kerbau daripada Kumpulan 5 yang menunjukkan perubahan kecil pada tanda klinikal dan mampu bertahan untuk 21 hari. Keputusan darah dan biokimia menunjukkan peningkatan sel darah putih, monosit dan eosinofil dalam kumpulan kerbau ini. Hanya paru-paru dan hati terjejas dengan sedikit perubahan histopatologi. Sama dengan Kumpulan 2, kerbau daripada Kumpulan 6 mempamerkan respon klinikal penyakit hawar berdarah dimana kerbau-kerbau tersebut hanya mampu bertahan selama 72 jam selepas jangkitan. Keputusan darah dan biokimia menunjukkan peningkatan monosit dan gamma glutamyl transferase yang tinggi. Semua organ-organ penting, pencernaan dan imunisasi terjejas teruk dengan perubahan histopatologi yang sederhana. Sebaliknya, tidak ada tanda klinikal yang ketara dalam kerbau daripada Kumpulan 7. Semua kerbau menunjukkan peningkatan monosit sepanjang 21 hari kajian. Hanya paru-paru dan hati terjejas dengan perubahan histopatologi yang sederhana. Kerbau dalam semua kumpulan rawatan P. *multocida* jenis B:2, lipopolisakarida dan membrane protin luar menunjukkan peningkatan dalam kepekatan haptoglobin dan serum amyloid A sepanjang tempoh kajian. Terdapat perbezaan yang signifikan ($p < 0.05$) dalam semua kumpulan rawatan berbanding kumpulan kawalan. Di samping itu, kerbau dalam semua kumpulan rawatan juga menunjukkan peningkatan yang ketara dalam kepekatan IgM, IgG dan IgA sepanjang tempoh kajian. Terdapat perbezaan yang signifikan ($p < 0.05$) dalam semua kumpulan rawatan berbanding kumpulan kawalan. Begitu juga, semua kumpulan rawatan menunjukkan peningkatan yang signifikan ($p < 0.05$) dalam kepekatan IL-2 dan IL-6; tetapi tidak menunjukkan signifikasi ($p > 0.05$) dalam kepekatan IL-12 berbanding kumpulan kawalan.

Daripada kajian ini, dapat disimpulkan bahawa kerbau dijangkiti dengan bakteria P. *multocida* jenis B:2, lipopolisakarida dan membran protin luar menunjukkan tindak balas sel yang berbeza. Maklumat mengenai ini akan memainkan peranan penting dalam memahami tindak balas sel badan semasa wabak hawar berdarah. Berdasarkan perubahan klinikal, darah, bedah siasat, histopatologi, dan kepekatan antibodi; kerbau disuntik secara oral dengan membran protin luar menunjukkan response yang positif dimana ia mungkin boleh dikembangkan untuk menjadi vaksin secara oral. Dengan ini, kita akan dapat mencari jalan alternatif untuk membuat vaksinasi secara menyeluruh untuk penyakit hawar berdarah. Selain itu, pembangunan penanda bio menggunakan sitokin pada masa akan datang dapat membantu mengawal penyakit hawar berdarah yang member kesan yang besar kepada ekonomi negara secara lebih efektif.

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

Faez Firdaus Jesse Abdullah, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Abd Wahid Haron, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

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

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

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Universiti Putra Malaysia

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LIST OF ABBREVIATIONS

HS	Haemorrhagic septicaemia
P. *multocida*	*Pasteurella multocida*
LPS	Lipopolysaccharide
OMP	Outer membrane protein
cfu	Colony forming unit
PCV	Packed cell volume
MCV	Mean corpuscular volume
MCHC	Mean corpuscular haemoglobin concentration
GGT	Gamma glutamyl transferase
A:G	Albumin:Globulin ration
Hp	Haptoglobin
SAA	Serum amyloid A
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-12	Interleukin-12
PM	Post mortem lesion
APP	Acute phase proteins

CHAPTER 1

INTRODUCTION

1.1 Introduction

P. *multocida* was first shown to be the causative agent of fowl cholera by Louis Pasteur in 1881. Since then, this Gram-negative bacterium had been identified as the causative agent of many other economically important diseases in a wide range of hosts. HS is a disease, caused by particular serotypes of P. *multocida* that causes acute, highly fatal septicaemia with high morbidity and mortality in cattle and more susceptible in buffaloes. Epidemic HS is caused by 1 or 2 serotypes of P. *multocida*, designated B:2 and E:2. E:2 causes outbreaks only in African region; however, B:2 serotype causes disease in Asian region (OIE, 2008, OIE, 2009). The disease is distinctly different from some other pasteurelloses when pasteurella play only a secondary role. HS is thus defined as a specific form of pasteurellosis in cattle and buffaloes (DeAlwis, 1992).

P. *multocida* is a gram negative, short, ovoid, bipolar staining coccoid forms. It is an extracellular parasite, and its immunity is primarily humoral. LPS, OMP, capsule, frimbae, iron and toxin are the few virulence factors of P. *multocida* type B:2 (DeAlwis, 1992). The cell envelope of Gram-negative bacteria consists of an inner cytoplasmic membrane, a thin peptidoglycan cell wall, and LPS containing OMP that surrounds the peptidoglycan layer (Quan et al., 2013). LPS can be found on the outer cell wall of the organism (Bain et al., 1982; Srivastava, 2013). LPS is released during multiplication which leads to inflammatory reaction. It represents the endotoxin of P. *multocida* type B:2 and responsible for toxicity in HS which plays an important role in the pathogenesis of the disease (Rhoades, 1967; Rebers, 1988; DeAlwis, 1999). After inhalation or oral ingestion, LPS causes endothelial damage enabling the entry of the P. *multocida* organism into the organs of susceptible cattle and buffaloes (DeAlwis, 1999; Zamri, 2013). The bacteria will then move into the circulation causing bacteraemia, septicaemia and then toxaemia. However, this theory has never been proven before. Experimental inoculation of LPS and OMP were found to cause clinico-pathological and haemato-biochemical changes in mice and cattle (Jesse et al., 2013; Jesse et al., 2013a; Ali et al., 2014).

P. *multocida* organism can survive for long hours and probably days in moist soil and water. This organism is transmitted by direct or indirect contact (Kahn and Line, 2005). In susceptible animals, septicaemia develops rapidly and causes death due to endotoxaemia within 8-24 hours after the development of the first sign. According to Abubakar et al. (2011), the heaviest loss occurs during stressor factors which include poor weather, humid conditions, high animal population density, extensive grazing system and poor husbandry practice in Southeast Asia. In most cases, the clinical findings are either acute or peracute, resulting death within 8 to 24 hours after onset (Kahn and Line, 2005). In an experiment done by DeAlwis (1992), buffaloes infected through aerosol and direct contact with diseased buffaloes showed clinical signs after 24 and 48 hours respectively. Animals first show sign of dullness, then reluctant to move, fever, salivation, and serous nasal discharge. Oedematous swelling is frequently seen, beginning at the throat region and then spreads to the parotid, neck and brisket region. Mucous membrane will then be congested leading to respiratory distress where

animals will usually die within hours (DeAlwis, 1999). For post mortem lesions, the most obvious lesions in affected animals are the oedema, widely distributed haemorrhages, and general hyperaemia. In most cases, there will also be clear or straw coloured oedematous fluid at the head, neck, brisket, and musculature region. Petechial haemorrhages are particularly prominent in the pharyngeal and cervical lymph nodes. Besides, blood tinge fluid is often found in the pericardial sac, thoracic and abdominal cavity (Kahn and Line, 2005; Abubakar et al., 2012).

Experiments done by Jesse et al. (2013d); Jesse et al. (2013e); and Abubakar et al. (2011), showed some interesting histopathology parameters which included pulmonary ooedema, presence of inflammatory cells, haemorrhage and necrosis in animals' organs after infecting P. *multocida* type B:2 via different routes of inoculations in mice, cattle and buffaloes. However, the signs observed following oral exposure were much milder compared to intratracheal or respiratory routes (Abubakar et al., 2012). This could be proof that oral route transmission could manipulate the P. *multocida* organism better compared to the other routes of inoculation. This indicated that the oral route could perhaps be a readily available route for effective vaccine administration and heightened the animals' immunity. Therefore, this study was done to know more about the clinico-pathological changes in buffaloes infected with P. *multocida* and its immunogens LPS and OMP infections via different routes of inoculations. Thus, this experiment will play a significant role in getting a better understanding and knowledge of the P. *multocida* organism and its immunogens.

According to Jesse et al. (2013a), inoculation of P. *multocida* type B:2 and its immunogens LPS and OMP stimulate response to the host humoral immunity and specific cellular response. During bacterial infection, haematological and biochemistry changes are first detected during routine blood sampling. However, animals' defence mechanism can react quite differently and there is no singular pattern in complete blood count that indicates a bacterial infection. Jesse et al. (2013b), had reported that there were some haematological and biochemical markers that could be used for early detection in animals infected with wild type of *Pasteurella multocida*. On the other hand, acute phase response will be the first line of immune defence mechanism against preliminary infections (Eckersall et al., 2008; Khaleel et al., 2013). APP such as fibrinogen, C-reactive, Hp, albumin and SAA are proteins found in the blood. These APP will either increase or decrease in cases of infection and inflammation (Jesse, et al., 2013c). Hepatic production of APP is stimulated by pro-inflammatory cytokines such as IL-1, IL-6 and tumour necrosis factor alpha that are released into the circulation during acute phase response (Eckersall et al., 2008).

Immunity is a process where the body protects against foreign organism or antigens (Blood et al., 2011). The immune system against HS is made of two important components which are the innate and humoral immunity. Innate immunity is also known as the first line of defence that prevents the entry of pathogens. Humoral immunity or the antibody-mediated immune system involves the production of specific antibodies against P. *multocida* which is an extracellular organism (Kumar, 2013). Morbidity and mortality due to HS in an endemic or epidemic area are largely dependent on the proportion of immune to non-immune animals. According to DeAlwis, (1999) there is also relation between cells mediated immunity and the

pathogenesis of P. *multocida* but is not explored yet. On the other hand, the production of humoral antibodies and cell mediated immunity are stimulated by the production of cytokines (Eckersall et al., 2008). IL-2 and IL-6 produced by macrophages and T-cells respectively are found to stimulate the production of humoral antibodies. Besides, macrophages are also found to produce IL-12 that will activate cell mediated immunity (Kindt et al., 2007).

1.2 Problem statement

1. Experimental infections of LPS and OMP via different routes of inoculations in the real host buffaloes have not been carried out before.
2. Information on clinical responses, post mortem, histopathological changes, haemato-biochemistry, APP, cytokines, as well as antibody titre in the real host buffalo after inoculation of LPS and OMP is still very scarce.
3. Outbreaks of HS were still reported despite vaccination and sudden death is usually the main finding in field animals during disease outbreak.

1.3 Hypotheses

It is hypothesized that there will be host cell response in buffaloes following experimental-infection with P. *multocida* type B:2 and its immunogens LPS and OMP via different routes of inoculation.

1. Buffaloes inoculated with P. *multocida* type B:2 will show more severe clinico-pathological changes compared to buffaloes inoculated with LPS and OMP via different routes of inoculation.
2. There will be changes in haemato-biochemistry, APP, IL, and antibody concentrations in the experimental animals infected with P. *multocida* type B:2 and its immunogens with LPS and OMP via different routes of inoculation.

1.4 Objectives

The main objective of this study was to determine the best candidate of P. *multocida* type B:2 and its immunogens LPS and OMP to be used as oral vaccination in buffaloes and cattle; together with the development of biomarkers to control outbreak of HS widely and more effectively. Thus, this study was further divided into 6 main objectives:

1. To determine the severity of the clinical response in experimental buffaloes infected with P. *multocida* type B:2 and its immunogens LPS and OMP via different routes of inoculation.
2. To determine and compare the blood and biochemistry changes in experimental buffaloes infected with P. *multocida* type B:2 and its immunogens LPS and OMP via different routes of inoculation.
3. To determine the severity of post mortem and histopathological changes in buffaloes infected with P. *multocida* type B:2 and its immunogens LPS and OMP via different routes of inoculation.

4. To determine and compare the concentration of APP in experimental buffaloes infected with P. *multocida* type B:2 and its immunogens LPS and OMP via different routes of inoculation.
5. To determine and compare the antibody titre in experimental buffaloes infected with P. *multocida* type B:2 and its immunogens LPS and OMP via different routes of inoculation.
6. To determine and compare the concentration of proinflammatory cytokines in experimental buffaloes infected with P. *multocida* type B:2 and its immunogens LPS and OMP via different routes of inoculation.

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