UNIVERSITI PUTRA MALAYSIA

RESPONSE OF RESPIRATORY, GASTROINTESTINAL, AND URINARY TRACTS OF BUFFALO CALVES FOLLOWING EXPOSURE TO PASTEURELLA MULTOCIDA B:2

ANNAS BIN SALLEH

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By

ANNAS BIN SALLEH

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

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May 2015

Chairman : Mohd Zamri Bin Saad, PhD
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Haemorrhagic septicaemia (HS) is an acute, fatal, septicaemic disease of ruminants, particularly in buffalo. HS is caused by a Gram-negative bacterium, *Pasteurella multocida* of specific serotypes; B:2 (Asian serotype) or E:2 (African serotype). Infection results in outbreaks and death of animals, which in turn leads to economic losses to the farmers. Animals surviving the outbreaks usually gain immunity and become life-long carriers. These carrier animals are believed to shed *P. multocida* B:2 via the respiratory tract, transmitting the organism to the surrounding naive animals, resulting in yet another outbreak and formation of new carrier animals. It has been long proven that the respiratory tract plays an important role in the transmission of HS among animals. However, since HS is a septicaemic disease, the aetiological agent could be isolated from all organs at necropsy and recent study revealed a new theory of the involvement of gastrointestinal and urinary tracts in development or transmission of HS. Therefore, this study was conducted to determine the involvement of gastrointestinal and urinary tracts in development of acute HS as well as its transmission especially among carrier animals.

Six buffalo calves were selected and divided into two groups. Group 1 was inoculated subcutaneously with 0.02 ml/kg of $1x10^9$ cfu/ml of *P. multocida* B:2, while Group 2 was subcutaneously inoculated with 0.02 ml/kg of sterile phosphate buffer saline (PBS) and served as the negative control group. The buffalo calves were observed for clinical signs of HS. All buffalo calves of Group 1 were euthanised due to advanced clinical signs, while all buffalo calves of Group 2 survived and were euthanised at 72 h p.i.. At necropsy, the organs of the respiratory, gastrointestinal, and urinary tracts were collected and subjected to *P. multocida* B:2 isolation

and concentration determination, as well as immunoperoxidase. The present study observed that the distribution of *P. multocida* B:2 based on the bacterial concentration and immunoperoxidase staining differs between the respiratory, gastrointestinal, and urinary tracts. In general, the distribution of *P. multocida* B:2 was observed to be significantly (p<0.05) high in the respiratory organs. However, the distribution and concentration of *P. multocida* B:2 in the gastrointestinal and urinary tracts, particularly in the liver, the small intestinal segments, and the kidneys were observed to be high. Severity of the pathological changes in these tracts was also compared. As expected, the lesions were most severe among the organs of the respiratory tract following gross, histopathological, and ultrastructural evaluations.

The involvement of respiratory, gastrointestinal, and urinary tracts in transmission of HS from carriers was determined in this study. 12 buffalo calves were selected and equally divided into three groups; Group 1 served as acute infection group, Group 2 as commingling group, and Group 3 as negative control group. Buffalo calves of Group 1 were inoculated subcutaneously with 0.02 ml/kg of $1x10^5$ cfu/ml of *P. multocida* B:2. Buffalo calves of Group 2 were not inoculated, but were allowed to commingle with buffalo calves of Group 1. Buffalo calves of Group 3 were inoculated subcutaneously with 0.02 ml/kg of sterile PBS. All buffalo calves were observed for clinical signs of HS, and all buffalo calves of Group 1 were euthanised at 24 to 48 h p.i., and transmitted the disease to the buffalo calves of Group 2, resulting in 3 buffalo calves to become carriers, while another had to be euthanised due to acute HS. The carrier animals of Group 2 and the negative control buffalo calves of Group 3 were subsequently subjected to three cycles of stress and immunosuppression by intramuscular injection of dexamethasone. At the end of the three cycles of immunosuppression, the carrier buffalo calves of Group 2, and the negative control buffalo calves of Group 3 were euthanised. At necropsy, samples of the respiratory, gastrointestinal, and urinary tracts were collected, and subjected to isolation and identification of *P. multocida* B:2, detection of *P. multocida* B:2 DNA by polymerase chain reaction (PCR), and immunoperoxidase for localisation of *P. multocida* B:2. Under the first cycle of immunosuppression, the carrier animals were observed to shed *P. multocida* B:2 via the respiratory, gastrointestinal, and urinary tracts following isolations of the organism from the nasal, rectal, and vaginal swabs. The immunoperoxidase technique was used to aid in localisation of *P. multocida* B:2 in respiratory, gastrointestinal, and urinary tracts of carrier animals. *Pasteurella multocida* B:2 was observed to localised in various organs of the respiratory, gastrointestinal, and urinary tracts. On the other hand, *P. multocida* B:2 DNA was detected in the tonsil, lungs, reticulum, ileum, and ureter of the carrier animals of Group 2.

Nine buffalo calves and nine cattle calves were selected to compare the susceptibility between buffalo and cattle calves upon exposure to *P. multocida* B:2. The animals were divided into six groups. Group 1 and Group 2 consist of three buffalo calves, and three cattle calves, respectively. These groups were inoculated subcutaneously with 0.02 ml/kg of sterile PBS and served as the negative control groups. Group 3 and Group 4 consisted of three buffalo calves, and three cattle calves, respectively. These groups were inoculated subcutaneously with 0.02 ml/kg of $1x10^5$ cfu/ml of *P. multocida* B:2. Group 5 and Group 6 consisted of three buffalo calves, and three cattle calves, respectively. These groups were inoculated intranasally with 0.02 ml/kg of $1x10^5$ cfu/ml of *P. multocida* B:2. Subsequently, samples of observation and recording of clinical signs severity, whole blood for quantitation of bacteraemia, and blood plasma for quantitation of endotoxaemia were collected. Animals with advanced clinical signs were euthanised. It was found that all buffalo and cattle calves of Group 3 and 4 and 2 buffalo calves of Group 5 had to be euthanised due to severe clinical signs of HS, pathological changes, and septicaemia. On the other hand, all cattle calves of Group 6 survived, and were euthanised at 72 h p.i.. Blood endotoxin and *P. multocida* B:2 concentrations throughout the experiment revealed that endotoxaemia preceeded bacteraemia prior to the development of septicaemia. Thus, it was postulated that the respiratory immunophysiology of cattle might contribute to its resistance to HS.

Based on high concentration of *P. multocida* B:2 in the lungs, liver, duodenum, jejunum, ileum, and kidney; high severity in scores in the lungs, abomasum, duodenum, jejunum, ileum, and kidney; isolation of *P. multocida* B:2 from the nasal, rectal and vaginal swabs of carrier animals; immunoreaction and *P. multocida* B:2 DNA detection from various organs of the respiratory, gastrointestinal, and urinary tracts of carrier animals; it was concluded that the respiratory, gastrointestinal, and urinary tracts play roles in the development and transmission of HS, although the respiratory tract remained as the most important system in HS transmission and development.

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TINDAK-BALAS SALURAN PERNAFASAN, GASTROUSUS DAN URINARI ANAK KERBAU AKIBAT PENDEDAHAN KEPADA *PASTEURELLA MULTOCIDA* B:2

Oleh

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Hawar berdarah (HS) ialah satu penyakit yang akut dan boleh membunuh haiwan ruminan, terutamanya kerbau melalui septisemia. HS disebabkan oleh bakteria Gram-negatif, *Pasteurella multocida* iaitu yang berserotip khusus, B:2 (serotip Asia) atau E:2 (serotip Afrika). Jangkitan menyebabkan letusan wabak dan kematian haiwan yang seterusnya menyebabkan kerugian ekonomi kepada peladang. Haiwan yang terselamat daripada letusan wabak berkenaan selalunya akan memperoleh imuniti dan menjadi haiwan pembawa sepanjang hayat. Haiwan-haiwan pembawa dipercayai akan mebebaskan *P. multocida* B:2 melalui saluran pernafasan, lalu akan merebakkan organisma ini kepada haiwan-haiwan yang naïf di sekelilingnya, akan menyebabkan wabak berlaku dan pembentukan haiwan pembawa yang baru. Dipercayai bahawa saluran pernafasan memainkan peranan yang penting dalam jangkitan penyakit HS. Bagaimanapun, oleh kerana HS merupakan penyakit septisemia, jadi, agen penyebab penyakit ini boleh dipencilkan dari semua organ semasa nekropsi dan daripada hasil kajian baru-baru ini mendedahkan teori baharu di mana terdapat kemungkinan penglibatan saluran gastrousus dan urinari dalam pembentukan dan penyebaran penyakit HS. Maka, kajian ini dijalankan bagi menentukan penglibatan saluran gastrousus dan urinari dalam pembentukan penyakit HS yang akut serta jangkitan terutamanya dalam haiwan pembawa.

Enam ekor anak kerbau telah dipilih, dan dibahagikan kepada dua kumpulan. Kumpulan 1 diinokulatkan secara subkutaneus dengan 0.02 ml/kg $1x10^9$ unit pembentukan koloni (cfu)/ml *P. multocida* B:2, sementara Kumpulan 2 telah diinokulat dengan 0.02 ml/kg salina penimbal fosfat (PBS) yang steril dan dijadikan sebagai kumpulan kawalan negatif.

Kesemua anak kerbau ini diperhatikan bagi mengesan tanda-tanda klinikal HS. Semua anak kerbau dari Kumpulan 1 telah dieutanasia disebabkan tanda klinikal yang teruk, sementara anak kerbau yang hidup dari Kumpulan 2 hidup dan telah dieutanasia pada 72 jam pi. Semasa nekropsi, organ-organ saluran pernafasan, gastrousus, dan urinari telah disampel, dan menjalani pemencilan *P. multocida* B:2 dan penentuan kepekatan, dan juga imunoperoksidase. Kajian ini mendapati bahawa taburan *P. multocida* B:2 melalui penentuan kepekatan bakteria dan imunoperoksidase berbeza di antara saluran pernafasan, gastrousus, dan urinari. Secara umumnya, taburan *P. multocida* B:2 didapati tertinggi dengan sangat berbeza (p<0.05) dalam organ-organ pernafasan. Walaubagaimanapun, taburan dan kepekatan *P. multocida* B:2 dalam saluran gastrousus dan urinari, terutamanya hati, dan usus kecil, serta buah pinggang didapati tinggi. Keparahan dalam perubahan patologi organ-organ dalam semua saluran tersebut telah dibandingkan. Seperti yang dijangkakan, keparahan lesi telah didapati paling tinggi di dalam organ-organ saluran pernafasan selepas penilaian secara kasar, histopatologi, dan ultrastruktur.

Penglibatan saluran pernafasan, gastrousus, dan urinari dalam penyebaran penyakit HS dari haiwan pembawa telah dikenalpasti di dalam kajian ini. 12 ekor anak kerbau telah dipilih, dan dibahagikan sama rata kepada 3 kumpulan; Kumpulan 1 dijadikan sebagai kumpulan jangkitan akut, Kumpulan 2 sebagai kumpulan campuran, dan Kumpulan 3 sebagai kumpulan kawalan negatif. Anak kerbau dari Kumpulan 1 telah diinokulatkan melalui subkutaneus dengan 0.02 ml/kg $1x10^5$ cfu/ml *P. multocida* B:2. Anak kerbau dari Kumpulan 2 tidak diinokulat, tetapi dibiarkan untuk untuk bercampur dengan anak kerbau dari Kumpulan 1. Anak kerbau dari Kumpulan 3 diinokulat melalui subkutaneus dengan 0.02 ml/kg PBS yang steril. Kesemua anak kerbau ini diperhatikan bagi mengesan tanda-tanda klinikal HS, dan kesemua anak kerbau dari Kumpulan 1 telah dieutenasia pada 24 hingga 48 jam p.i., dan telah menjangkitkan anak kerbau Kumpulan 2. Ini menyebabkan 3 anak kerbau dari Kumpulan 2 menjadi haiwan pembawa, manakala seekor terpaksa dieutenasia kerana HS akut. Haiwan pembawa dari Kumpulan 2, dan anak kerbau kawalan negatif dari Kumpulan 3 kemudiannya dihadapkan dengan tiga kitaran stres dan imunotindasan dengan suntikan dexamethasone secara intraotot. Pada akhir kitaran imunotindasan, haiwan pembawa dari Kumpulan 2, dan anak kerbau kawalan negatif dari Kumpulan 3 telah dieutenasia. Semasa nekropsi, sampel-sampel dari saluran pernafasan, gastrousus, dan urinari telah dipungut, dan menjalani pemencilan *P. multocida* B:2, pengesanan DNA *P. multocida* B:2 menggunakan reaksi rantai polymerase (PCR), dan imunoperoksidase untuk penempatan *P. multocida* B:2. Semasa kitar imunotindasan yang pertama, haiwan-haiwan pembawa didapati telah membebaskan *P. multocida* B:2 melalui kesemua saluran pernafasan, gastrousus, dan urinari, di mana organisma ini berjaya dipencilkan melaui calitan hidung,

rektum, dan vagina. Teknik imunoperoksidase telah digunakan bagi membantu mengesan *P. multocida* B:2 di dalam saluran pernafasan, gastrousus, dan urinari haiwan-haiwan pembawa tersebut. *Pasteurella multocida* B:2 telah diperhatikan untuk menyetempatkan di dalam pelbagai organ saluran pernafasan, gastrousus, dan urinari haiwan-haiwan pembawa dari Kumpulan 2. DNA *P. multocida* B:2 telah dikesan di dalam tonsil, peparu, reticulum, ileum, dan ureter haiwan-haiwan pembawa dari Kumpulan 2.

Sembilan anak kerbau dan Sembilan anak lembu dipilih bagi membandingkan kerentanan di antara anak kerbau dan anak lembu selepas jangkitan oleh *P. multocida* B:2. Haiwan-haiwan ini dibahagikan kepada enam kumpulan. Kumpulan 1 dan Kumpulan 2 terdiri daripada tiga ekor anak kerbau, dan tiga ekor anak lembu, masing-masing. Kumpulan-kumpulan ini telah diinokulat secara subkutaneus dengan 0.02 ml/kg PBS yang steril dan dijadikan sebagai kumpulan kawalan negatif. Kumpulan 3 dan Kumpulan 4 terdiri daripada tiga anak kerbau, dan tiga anak lembu, masing-masing. Kumpulan-kumpulan ini diinokulat secara subkutaneus dengan 0.02 ml/kg $1x10^5$ cfu/ml *P. multocida* B:2. Kumpulan 5 dan Kumpulan 6 terdiri daripada tiga anak kerbau, dan tiga anak lembu, masing-masing. Kumpulan-kumpulan ini diinokulat secara intranasal dengan 0.02 ml/kg $1x10^5$ cfu/ml *P. multocida* B:2. Kemudian, sampel pemerhatian dan merekod keterukan tanda-tanda klinikal, darah penuh bagi mengkuantitasi bakteremia, dan plasma darah bagi mengkuantitasi endotoksemia telah dipungut. Haiwan-haiwan dengan tanda-tanda klinikal lanjutan telah dieutenasia. Didapati bahawa kesemua anak kerbau dan anak lembu dari Kumpulan 3 dan 4, dan 2 anak kerbau dari Kumpulan 5 terpaksa dieutenasia akibat HS dengan tanda-tanda klinikal, perubahan patologi, serta septisemia yang teruk. Dalam pada itu, kesemua anak lembu dari Kumpulan 6 yang dijangkiti secara intranasal terselamat, dan dieutenasia pada 72 jam p.i.. Hasil perubahan kepekatan endotoksin dan *P. multocida* B:2 dalam darah sepanjang eksperimen ini menyaksikan bahawa endotoksemia berlaku terlebih dahulu sebelum bakteremia semasa pembentukan septisemia. Dengan itu, telah dipostulatkan bahawa sifat-sifat imunofisiologi pernafasan lembu mungkin menyumbang kepada ketahanannya terhadap HS.

Berdasarkan kepekatan *P. multocida* B:2 yang tinggi di dalam peparu, duodenum, jejunum, ileum, dan buah pinggang; skor keterukan yang tinggi di dalam peparu, abomasum, duodenum, jejunum, ileum, dan buah pinggang; pemencilan *P. multocida* B:2 dari calitan nasal, rectum dan vagina dari haiwan-haiwan pembawa; tindak balas imun dan pengesanan DNA *P. multocida* B:2 dari pelbagai organ saluran pernafasan, gastrousus, dan urinari dari haiwan-haiwan pembawa; disimpulkan bahawa kesemua saluran pernafasan, gastrousus, dan kencing memainkan peranan dalam pembentukan dan penyebaran HS,

walaubagaimanapun, saluran pernafasan kekal sebagai sistem utama dalam penyakit HS.

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

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µl	microliter
µm	micrometer
ºC	degree Celcius
ANOVA	analysis of variance
BALT	bronchus-associated lymphoid tissue
BHI	brain-heart infusion
BSA	bovine serum albumin
cfu	colony forming unit
DAB	3,3'-Diaminobenzidine
df	degree of freedom
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EU	endotoxin unit
G	gravity
g	gram
h	hour
HE	haematoxylin and eosin
HS	haemorrhagic septicaemia
IACUC	Institutional Animal Care and Use Committee
IgG	immunoglobulin G
IL	interleukin
IP	immunoperoxidase
LAL	limulus amebocyte lysate
LPS	lipopolysaccharide
LRT	lower respiratory tract
M	molar
min	minute
ml	milliliter
n	sample size
ng	nanogram
nm	nanometer
OMP	outer membrane protein
p.i.	post-inoculation
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with tween 20
PCR	polymerase chain reaction
PPP	platelet poor plasma
r^2	coefficient of determination
RBC	red blood cell
rpm	revolution per minute
SD	standard deviation
SEM	standard error of the mean

SPSS	Statistical Packages for the Social Sciences
TBE	tris-boric acid-EDTA
TEM	transmission electron microscopy
TNF-α	tumour necrosis factor-α
URT	upper respiratory tract
V	volt

CHAPTER 1

INTRODUCTION

Haemorrhagic Septicaemia (HS) is an acute, fatal, septicaemic disease of cattle and buffaloes, causing devastating epidemics with high morbidity and mortality especially in the South and South-East Asia, Africa, and some South European and Middle Eastern countries (De Alwis, 1992; Verma and Jaiswal, 1998; Benkirane and De Alwis, 2002). In South-East Asia, HS occurs in Malaysia, Indonesia, Phillipines and Thailand (De Alwis, 1992). In Japan, HS was recognised in 1923 but has not been reported since 1954 (De Alwis, 1999). The disease has been reported in the USA among American Bison in 1912, 1922 and 1967, and among dairy cattle in 1969 and beef calves in 1993 (De Alwis, 1992; Verma and Jaiswal, 1998; De Alwis, 1999).

Pasteurella multocida serotype B:2 (known as the Asian serotype) and E:2 (known as the African serotype) by Carter-Heddleston system which correspond to 6B and 6E by Namioka-Carter system are the specific serotypes of bacteria known to cause HS in ruminants. In North America, an HS outbreak was presumed to be caused by serotype B:2 until a re-examination revealed that it was in fact caused by serotype B:3 and B:4 (Rimler and Wilson, 1994). Kumar *et al*. (1996) also described the presence of other serotypes causing HS-like condition and lesions in cattle and buffaloes, mostly by A:1 and A:3.

Pasteurella multocida B:2 is a Gram-negative bacterium. Being a Gram-negative bacteria, the bacterial cell wall consist of components which act as virulence factors, such as the capsule, lipopolysaccharide (LPS) (endotoxin), fimbriae, adhesins, and outer membrane protein (OMP) (Harper *et al*., 2006). Endotoxaemia has been recognised as an important process in the development of acute HS (Horadagoda *et al*., 2001; Horadagoda *et al*., 2002). Previous study involving the inoculation of lipopolysaccharide (LPS) intravenously resulted in comparable pathological lesions in both field and experimentally-induced HS (Horadagoda *et al*., 2002).

In general, upon exposure to the aetiological agent, there are two possible outcomes; the animal would succumb to peracute or acute HS or the animal would survive the infection and become carriers harbouring *P. multocida* B:2 (De Alwis *et al*., 1995). In peracute or acute HS, the disease is characterised by a short clinical course (Biswas *et al*., 2004; Zamri-Saad and Shafarin, 2007) with clinical signs such as severe

depression, pyrexia, submandibular oedema, dyspnoea, recumbency, and death (Horadagoda *et al*., 2001; Zamri-Saad and Shafarin, 2007). If the animal survived the initial infection and became carrier, the animal would exhibit minimal clinical signs that are easily overlooked, such as transient pyrexia and mild depression (De Alwis, 1999). The persistence of *P. multocida* B:2 in carriers lead to difficulty in control and prevention of new outbreak (Townsend *et al*., 2000).

Infections are believed to occur by inhalation and/or ingestion of the aetiological agent (Saharee *et al*., 1993; Benkirane and De Alwis, 2002) since *P. multocida* B:2 has been isolated in both the nasopharynx and intestine of dead cattle and buffaloes (Khin *et al*., 2010a; Abubakar *et al*., 2012). The respiratory tract may not be the only portal of entry, and circumstantial evidence suggest involvement of other routes such as the gastrointestinal tract (Zamri-Saad and Shafarin, 2007; Abubakar and Zamri-Saad, 2011).

Higher incidence of HS is associated with stress conditions such as high moisture environment, humid conditions, high animal stocking density, extensive free grazing system, inclement weather, transportation, and poor husbandry practice (Benkirane and De Alwis, 2002; Zamri-Saad and Shafarin, 2007). These stress factors are believed to contribute to conversion of latent carrier to active carriers, which eventually leads to transmission of the aetiological agent to susceptible in-contact animals, leading to outbreaks (De Alwis *et al*., 1990; Shafarin *et al*., 2007).

Among the carrier animals, many studies observed that shedding of *P. multocida* B:2 occur mainly via the respiratory route, where the organism is most frequently isolated in the nasopharynx of active carriers by deep nasal swabbing (Singh, 1948; Mohan *et al*., 1968; Hiramune and De Alwis, 1982; De Alwis *et al*., 1990). However, recent findings in acutely infected animals suggested that the shedding of the aetiological agent is via the gastrointestinal and urinary tract (Abubakar and Zamri-Saad, 2010; Abubakar *et al*., 2012). However, the involvement of the whole respiratory, gastrointestinal, and urinary tracts in transmission of HS involving the carrier animals has never been documented. Therefore, the research hypotheses and objectives are as follows:

1.1 Research hypotheses

1. Distribution of *P. multocida* B:2 and pathological changes in the respiratory, gastrointestinal, and urinary tracts of buffalo calves following exposure to live *P. multocida* B:2 are comparable.
2. The gastrointestinal and urinary tracts play a role in retention of *P. multocida* B:2 and transmission of HS in carrier animals.
3. Clinicopathological changes, development and susceptibility of cattle and buffalo calves to HS are similar.

1.2 Objectives of the study

1. To determine the distribution of *P. multocida* B:2 in the respiratory, gastrointestinal, and urinary tracts of buffalo calves, following acute infection.
2. To observe and define the pathological changes of the respiratory, gastrointestinal, and urinary tracts of buffalo calves following acute *P. multocida* B:2 infection.
3. To determine the role of carrier animals in retention of *P. multocida* B:2 within the gastrointestinal and urinary tracts.
4. To compare the clinicopathological changes, development of, and susceptibility to HS in cattle and buffalo calves following experimental exposure to *P. multocida* B:2.

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