UNIVERSITI PUTRA MALAYSIA

PURIFICATION AND IMMUNOLOGICAL EVALUATION OF PROTECTIVE ANTIGENS OF OUTER MEMBRANE ORIGIN OF *PASTEURELLA MULTOCIDA* TYPE 6: B

RAMLAN MOHAMED

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By

RAMLAN MOHAMED

Thesis Submitted in Fulfilment of the Requirements for the Degree of Master of Science in the Faculty of Veterinary Medicine Universiti Putra Malaysia

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PURIFICATION AND IMMUNOLOGICAL EVALUATION OF PROTECTIVE ANTIGENS OF OUTER MEMBRANE ORIGIN OF *PASTEURELLA MULTOCIDA* TYPE 6:B

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October 2000

Chairman : Associate Professor Rasedee bin Abdullah, Ph.D.

Faculty : Veterinary Medicine

A study on the toxic effects of lipopolysaccharide (LPS) of *Pasteurella multocida* type 6:B in mice was conducted. In addition, the immunogenic potential of LPS as subunit vaccines was evaluated in mice. Initial studies showed that the purified LPS, which contained 0.14% protein as contaminant, had toxic effects in mice even at low levels. The mice exhibited toxic effects such as depression and ruffled hair coat after a few hours of inoculation and they survived if limited inoculation doses were used. It could be speculated that the protection afforded in mice immunised with LPS was not due

to the LPS and the protection could be attributed to the contamination of proteins in the LPS extracts.

Protective capacities of whole-cell killed vaccines grown under iron-regulated conditions were evaluated. Vaccines made from formalin-killed whole cells of *P. multocida* type 6:B grown under iron-restricted conditions were found to be superior in imparting protection to mice against experimental pasteurellosis than those grown under iron-repleted conditions. It could be speculated that some cell-associated antigens responsible for protective immunity were expressed better under iron-limiting conditions. The isolation and purification of outer membrane proteins (OMPs) in this study was from *P. multocida* grown under iron-restricted condition where α,α'-dipyridyl was used as the iron chelator.

The extraction of OMP of *P. multocida* was carried out using Sarkosyl method. The final purified OMP was chosen for subsequent purification studies, as it contained less LPS and afforded 100% protection to immunised mice. The extracts were shown to have about 12 protein bands when electrophoressed on SDS-PAGE gels in which 33 kD and 37 kD proteins appeared as major OMPs of *P. multocida* with the 87 kD and 116 kD protein bands as next prominent ones.

Active protection studies of purified proteins obtained after preparative electrophoresis using Prep Cell, cylindrical and stained gels have shown that proteins with MWs 29, 33 and 37 kD have the potential as subunit vaccines. Whereas, high MW proteins only afforded up to 60% protection to the immunised mice. However, the immune response of the target and laboratory animals was different as judged by

comparative immunoblotting. Almost all the antibodies of mice and cattle antiserum reacted to 29, 33, 37, 52, 71, 87 and 116 kD proteins except 33 kD protein for cattle antiserum. It showed that the mice immune response is very similar to the cattle.

Mice immunised with purified 29 kD alone was shown to afford a lower protection. When it was excised, electro-eluted then rerun on mini SDS-PAGE, the 29 kD protein produced a ladder form of protein bands of MW 29, 37 and 47 kD. It was speculated that the 29 kD protein is a monomeric form of the protein H of *P. multocida* and the 37 kD protein is a trimer. Thus, the trimeric form with MW 37 kD protein could be an attractive vaccine candidate.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

PENULINAN DAN PENILAIAN SECARA IMMUNOLOGI ANTIGEN YANG PROTEKTIF DARI MEMBRANE LUAR BERASAL DARI *PASTEURELLA MULTOCIDA* JENIS 6:B

Oleh

RAMLAN MOHAMED

Oktober 2000

Pengerusi : Profesor Madya Rasedee bin Abdullah, Ph.D.

Fakulti : Perubatan Veterinar

Suatu kajian mengenai kesan-kesan keracunan lipopolisakarid dari bakteria *P. multocida* jenis 6:B terhadap tikus telah dilakukan. Selain daripada itu potensi LPS yang mempunyai daya pelindungan (immunogenic) sebagai subunit ubat pelalian telah dinilai didalam tikus. Diawal kajian menunjukkan bahawa LPS tulin dengan kandungan protin 0.14% sebagai bahan sampingan mempunyai kesan-kesan keracunan seperti depression dan bulu-bulu yang kusut beberapa jam selepas suntikan dan adalah selamat sekiranya suntikan pelalian dilakukan secara berperingkat-peringkat. Didalam kajian ini juga adalah dicadangkan bahawa daya

perlindungan yang diterima oleh tikus yang diberi pelalian LPS, bukan disebabkan perlindungan dari LPS. Perlindungan yang diterima oleh tikus melawan pasteurellosis berkemungkinan adalah disebabkan bahan sampingan protin.

Keupayaan dalam memberi perlindungan oleh ubat pelalian yang terdiri dari sel-sel mati bakteria yang tumbuh dibawah keadaan kawalan ion ferum adalah dinilai. Didapati bahawa ubat pelalian yang dibuat daripada sel-sel mati bakteria *P. multocida* yang tumbuh dibawah keadaan kekurangan ion ferum telah menyumbang/memberi daya perlindungan yang lebih baik berbanding yang tumbuh dalam keadaan yang diperkaya dengan ion ferum. Ini menunjukkan bahawa antigen yang terdapat pada sel bakteria dan bertanggungjawab untuk memberi perlindungan telah dijanakan/dihasilkan lebih baik dibawah keadaan kekurangan ion ferum. Pengasingan dan penulinan protin-protin membran luar (OMPs) didalam kajian ini telah menggunakan *P. multocida* yang tumbuh didalam keadaan kekurangan ion ferum dengan α,α'-dipyridyl digunakan sebagai pemerangkap ferum.

Ekstraksi OMP dari *P. multocida* dilakukan menggunakan kaedah Sarkosyl. Hasil akhir didalam proses penulinan OMP digunakan untuk kajian selanjutnya memandangkan ianya memberi perlindungan 100% terhadap tikus yang diberi pelalian dan terdapat kandungan LPS yang rendah. Didalam gel mini SDS-PAGE, ekstrak telah menunjukan terdapat lebih kurang 12 jaluran protin dimana protin-protin 33 kD dan 37 kD merupakan protin utama dalam OMP manakala protin-protin 87 dan 116 kD pula prominan.

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I certify that an Examination Committee met on 31st October 2000 to conduct the final examination of Ramlan Mohamed on his Master of Science thesis entitled "Purification and Immunological Evaluation of Protective Antigens of Outer Membrane Origin of *Pasteurella multocida* Type 6:B" in accordance with the 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:

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

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

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

Muniandy Narasiman, PhD
Senior Research Officer
Veterinary Research Institute
Department of Veterinary Services
(Member)

MOHD GHAZALI MOHAYIDIN, PhD
Professor Deputy Dean of Graduate School,
Universiti Putra Malaysia

Date: 28 NOV 2000

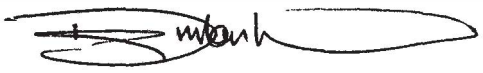
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

KAMIS AWANG, PhD
Associate Professor
Dean of Graduate School,
Universiti Putra Malaysia

Date: 11 JAN 2001

DECLARATION

I hereby declare that this thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



RAMLAN MOHAMED

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ABBREVIATIONS

AP	Alkaline phosphatase
APV	Alum-precipited vaccine
BA	Blood agar
BB	Broth bacterin
BCIP	Bromochlroindoyl phosphate
BHI	Brain heart infusion
BSA	Bovine serum albumin
Cetrimide	Cetyl trimethylammonium bromide
CFU	Colony forming unit
conc.	Concentration
DIP	α,α'-dipyridyl
DMSO	Dimethyl sulphoxide
DVS	Department of Veterinary Services
dwater	Distilled water
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
$FeCl_3$	Ferric chloride
g	Gram
HIO_4	Periodic acid
HS	Haemorrhagic septicaemia
i/p	Intraperitoneal
IgG	Immunoglobulin
IROMP	Iron-regulated outer membrane protein
kD	Kilodaltons
KDO	2-keto-3-deoxyoctonate
KSCN	Potassium thiocyanate
LPS	Lipopolysaccharide
M	Molar
m. liter	Million liter
m. tan	Metric tan
mAb	Monoclonal antibody
mg	Miligram
mins	Minutes
ml	Mililiter
mM	Milimolar
mm	Milimeter
MW	Molecular weight
N	Normal

$NaAsO_4$	Sodium arsenite
NBT	Nitro blue tetrazolium
nm	Nanometer
OAV	Oil adjuvant vaccine
OD	Optical density
OMP	Outer membrane protein
P. haemolytica	*Pasteurella haemolytica*
P. multocida	*Pasteurella multocida*
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-Tween
PMSF	Phenylmethysulphonyl fluoride
PMT	*Pasteurella multocida* toxin
PVDF	Polyvinylidene difluoride
rpm	Revolutions per minute
RT	Room temperature
s/c	Subcutaneously
s/p	Subperitoneal
sarkosyl	N-Lauroyl-Sarcosine
SBA	Sheep blood agar
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
TBA	Thiobarbituric acid
V	Volt
v/v/v	volume/volume/volume
VRI	Veterinary Research Institute
w/v	Weight/volume
µg	Microgram
µm	Micrometer

CHAPTER I

INTRODUCTION

The livestock industry is important in providing high quality protein for the population and raw materials for the food processing industry. The ruminant sector has registered steady growth largely due to greater participation of government land development agencies in cattle and sheep rearing integrated with plantation crops. In 1995, 15 per cent of the cattle population was raised through integration of cattle with plantation crops. In Malaysia, the average annual per capita consumption of both beef and mutton continued to increase by 6.0 and 4.1 per cent, respectively in 1985 and 1995. In terms of market potential, the domestic demand for fresh beef and mutton are expected to increase. The total projected demand for beef is 88,000 tonnes in 1995 and 240,000 tonnes by the year 2010 with per capita consumption for the same years being 4.3 kg and 8.4 kg, respectively (Third National Agricultural Policy, 1998 – 2010).

According to the 1995 livestock statistics compiled by the Department of Veterinary Services (DVS), the cattle and buffalo population in Malaysia ranged from about 765,000 to 892,000 heads for the last ten-year period (Table 1). These animals were used more for the provision of meat and milk. The beef consumption in the ten-year period has increased from 37,442 metric ton in 1986 to 86,039 metric ton in 1995 and the milk production was at an average of 68.27 million liter per year.

Table 1

Cattle and buffalo population and the livestock products in Malaysia: 1986 – 1996 reported by Department of Veterinary Services annual report.

Year	Cattle	Buffalo	Milk (m. liter)	Beef consumption (m. tonnes)
1986	607,180	206,629	538.02	37,442
1987	631,699	215,770	608.40	40,571
1988	640,621	213,145	762.73	46,381
1989	661,041	210,061	581.72	54,289
1990	652,498	194,517	561.18	57,424
1991	637,663	127,850	829.23	63,832
1992	654,358	122,259	792.23	65,467
1993	704,687	119,853	729.88	70,115
1994	724,353	167,853	535.65	77,699
1995	715,279	165,061	863.62	86,039

Haemorrhagic septicaemia (HS), an acute septicaemic disease principally affecting cattle and buffalo, is caused by two serotypes of *Pasteurella multocida*, viz., Asian serotype B:2 and the African serotype E:2 (Heddleston et al., 1972) corresponding to 6:B and 6:E respectively (Namioka and Murata, 1961). The disease is characterised by a rapid course, oedematous swelling in the head-throat-brisket region, swollen and haemorrhagic lymph nodes and the presence of numerous subserous petechial haemorrhages. Haemorrhagic septicaemia is distinctly different from other pasteurellosis where Pasteurellae may play only a secondary role. It is a specific form of pasteurellosis in cattle and buffaloes, just as typhoid fever and

pullorum are specific forms of salmonellosis in man and poultry, respectively. In cattle and buffaloes the condition is termed septicaemic pasteurellosis.

Although there is some reduction in the occurence of the disease due to the vaccination programmes, outbreaks of HS still occur at the beginning of the rainy season particularly in Asia (Bain *et al.*, 1982a). In many Asian countries, the disease has a great socio-economic significance to livestock farmers, particularly the small-scale subsistence farmers who depend on cattle and buffaloes for their farming activities. These animals are used not only for provision of meat and milk but also for draught power for agricultural purposes (Bain *et al.*, 1982b).

Haemorrhagic septicaemia in Malaysia has been known to occur for more than 90 years. The disease was first described in 1900 (Corongean, 1902). The disease has become enzootic in this country and continues to be a problem with the loss of susceptible livestock. Although there is no up to date data on losses due to this disease, it was estimated that approximately RM1.5 million worth of livestock was lost in West Malaysia in 1966. The Office International Des Epizooties (OIE) reported that for the period between 1992 to Jun 1997, infection with *P. multocida* was highest in India affecting more than one hundred thousand animals (see Table 2) followed by Vietnam (>13,000), Indonesia (>9,000), Myanmar (>3,000) and Sri Langka (971). In Malaysia the number of animal infected with *P. multocida* during that period was 453 animals (OIE Report, 1997).

It has been noted that many outbreaks occur during the monsoon and padi planting seasons in all states of Malaysia. Although the reasons for the outbreaks are not clear, the inclement weather and stress of work could probably lower the

resistance of the animals (Chandrasekaran, 1988). However, outbreaks can still occur at any time of the year.

Haemorrhagic septicaemia in Malaysia is controlled during outbreaks by prophylactic vaccination. Despite rigorous vaccination carried out by the DVS in 1988, 1989 and 1990 involving 134,965, 115,915 and 191,393 animals respectively, outbreaks of the disease still occur due to the short falls in vaccination resulting in 4, 216 and 163 mortalities, respectively. The outbreaks were reported in Alor Gajah Melaka, Rembau Negeri Sembilan, Maran and Kuala Nerus in Terengganu as well as in a government cattle farm at Ulu Lepar Pahang (DVS Annual Report 1988/89, DVS Annual Report 1990). The disease was successfully controlled through the use of vaccines (oil adjuvant and broth bacterin) produced by the Veterinary Research Institute (VRI) and no outbreaks were recorded for the years 1990 to 1995. The farmers then discontinued the vaccination at the end of 1995 with the assumption that HS was under control. However, in 1996 HS occurred in Seberang Prai Pulau Pinang and Negeri Sembilan at Rembau and Kuala Pilah districts where 12 cattle and 22 buffaloes died.

At present, the only means of effective control is by vaccination. Although considerable reduction in deaths due to this disease had been achieved by immunisation with the currently available vaccines, there are still some major problems including adverse reactions and other drawbacks encountered with the use of the vaccines. Vaccination with killed bacteria is practiced and is sometimes effective in controlling clinical disease. However, it is not uncommon for vaccinated herds to suffer outbreaks (Adler *et al.*, 1996). Moreover, immunity is generally

serotype specific. Empirically derived live virulent strains have been used as vaccines in both poultry and cattle (Derieux, 1984; Myint *et al.*, 1987), but the basis for attenuation is not known. It is therefore not surprising that reversion to virulence has occasionally occurred (Hofacre, 1986).

Three types of vaccines are commonly used in the field to control HS; the broth bacterin (BB), alum-precipitated vaccine (APV) and the more popular oil adjuvant vaccine (OAV). Broth bacterin is the first vaccine used in this country and had afforded protection for up to 3 months in cattle (Thomas, 1971). Initially only broth bacterin was used as a prophylactic agent against HS in Malaysia. However, between 1965 – 1966 there were reports of outbreaks of HS among vaccinated animals, which was attributed to 'vaccine breakdown' and thus initiated the shift to the usage of either APV or OAV. Broth bacterin had been used in Malaysia initially but was replaced by APV as animals vaccinated with broth bacterin showed symptoms of anaphylactic shock reaction within minutes of inoculation. The symptoms appeared several hours after vaccination and animals died from post vaccination shock if not treated with anti-histamine and adrenaline (Bain *et al.*, 1982a). Similar incidence of post-vaccination shock has been reported in many other contries (Bain *et al.*, 1982a).

The duration of immunity provided by the vaccine is still a problem. Many countries use the OAV, which has been claimed to impart a longer duration of immunity for up to a maximum of one-year (Chandrasekaran & Geneidy, 1994). In the countries like Sri Lanka, Indonesia and India, annual vaccination with OAV is practised, but in Thailand, Malaysia, Vietnam, Cambodia, Myanmar and Philippines,

the use APV is preferred because of its ease of production and administration. Alum-precipitated vaccine provides immunity for up to six months and two injections per year were needed. However, the usage of APV has become a problem for the countries where more than one enzootic disease exists when more than one vaccine cannot be administered simultaneously (Interior, 1993). The farmers usually do not want their cattle or buffaloes to be vaccinated many times (Neramitmansook, 1993).

Although OAV is a more popular vaccine in many countries because it induces a much longer immunity i.e up to one year, it is highly viscous and very difficult to administer intramuscularly due to its thick consistency. This makes it an unpopular product among vaccinators (Bain *et al*., 1982a). Improper administration of the vaccine also produces local tissue reactions resulting in large, painful and persistent subcutaneous swellings. The other major problems associated with the current vaccines are breakdown of immunity and lack of consistency in the quality of different batch of vaccines.

To overcome the problems associated with the use of currently available vaccines, the quality and effectiveness of vaccine need to be improved. One of the strategies to improve the effectiveness is by enhancing the capability of the vaccine to enhance protective antibody response. This could be achieved in a number of ways. Firstly, the growth conditions of the bacteria could be regulated or manipulated to allow the organism to express certain specific immunogens of high quality. Secondly, the most-potent immunogens of the organism need to be identified and subunit vaccines produced using these immunogens.

The experiments carried out in this study were designed to achieve the following objectives:

1. To determine the minimum moribund dose and the minimum time required for immune response in immunised mice.

2. To determine the toxic effects of lipopolysacharide in mice.

3. To identify and purify the most potent immunogens of *P. multocida* type B:6.

4. To evaluate the protective capacities of the killed whole-cells, LPS and OMPs of *P. multocida* type B:6 grown under iron-rich and iron-depleted conditions.

5. To identify the proteins of outer membrane origin which are expressed under iron-limiting condition.