UNIVERSITI PUTRA MALAYSIA

CLONING AND EXPRESSION OF FIMBRIAL SUBUNIT GENE OF PASTEURELLA MULTOCIDA TYPE 6:B, ISOLATED FROM CATTLE WITH HAEMORRHAGIC SEPTICAEMIA

ERNIE ZURAIDA BINTI ALI

FPV 2005 11

CLONING AND EXPRESSION OF FIMBRIAL SUBUNIT GENE OF *PASTEURELLA MULTOCIDA* TYPE 6:B, ISOLATED FROM CATTLE WITH HAEMORRHAGIC SEPTICAEMIA

By

ERNIE ZURAIDA BINTI ALI

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

June 2005

DEDICATED TO.....

My Father and Mother,

TUAN HJ. ALI ALIAS
PUAN HJH. SALNAH ALI

My Elder Sister,

ERNIE SUZANA ALI

My Sisters and Brothers,

NUR HAFIZAH ALI
SITI KHADIJAH ALI
MUHD. AMINUDDIN ANWAR ALI
MUHD FAIZ ALI

My Beloved Love,

MOHD AMRAN MOHD RADZI

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

CLONING AND EXPRESSION OF FIMBRIAL SUBUNIT GENE OF *PASTEURELLA MULTOCIDA* TYPE 6:B, ISOLATED FROM CATTLE WITH HAEMORRHAGIC SEPTICAEMIA

By

ERNIE ZURAIDA BINTI ALI

June 2005

Chairman: **Professor Mohd. Zamri Saad, PhD**

Faculty: **Veterinary Medicine**

Haemorrhagic septicaemia (HS) is a common disease of cattle and buffaloes, particularly in Asia. In Malaysia, *Pasteurella multocida* 6:B is most commonly isolated from outbreaks of haemorrhagic septicaemia. Thus, many antigenic components of *P. multocida* have been studied such as the lipopolysaccharides (LPS), outer membrane proteins (OMP) and the capsule. However, the fimbriae, which is involved in the attachment to the cell surface of the host and usually correlated with virulence of the organism has not been studied. Thus, studies on fimbrial gene and protein may be essential in the production of vaccine against haemorrhagic septicaemia.

In this study, fimbriae gene of *P. multocida* type 6:B was amplified, cloned and subjected for sequencing and expression in *Pseudomonas aeruginosa* and *Eshericheria coli*. All isolates produced a single product approximately at 450 bp. Analysis of the fimbrial subunit gene sequence of type 6:B strain was compared with those of type A:1 and A:3 strains of *P. multocida*. The sequence of strains A:3 and 6:B showed complete homology while the sequence of strains A:1 and 6:B showed

81.8% amino acid similarity. Although the *P. multocida* and other species shared that mean showed the conserved same mature fimbriae, both showed different signal peptides, even though they were within the same group/type.

Pasteurella multocida fimbrial subunit gene was cloned in the expression vectors, pUCpKS/SK and pCRT7-TOPO in order to construct a recombinant plasmid. In SDS-PAGE gel, it was seen that the recombinant *P. aeruginosa* cells failed to produce fimbriae using a specific surface fimbriae method. On Western blot analysis using anti-*P. aeruginosa* fimbrial antiserum, reaction was observed in both the wild type *P. aeruginosa* and the whole cells of recombinant *P. aeruginosa* cells. However, only the wild type *P. aeruginosa* showed cross-reaction when probed with anti-*P. multocida* fimbrial antiserum. This indicated that the wild type *P. aeruginosa* shared the same epitope with *P. multicoda* and that the fimbriae proteins of *P. multocida* was not expressed in *P. aeruginosa*.

In *E. coli* cells, the recombinant protein was expressed as a soluble protein but at a relatively low level despite optimization. In the Western blot analysis using anti-*P. multocida* fimbrial polyclonal antibody, the recombinant protein was identified as the protein band that have a molecular weight of approximately 18 kDa. However, it was uncertain whether the endogeneous fimbriae was not expressed or the protein was expressed but was not exported out of the cell. Thus, further analysis to identify the other candidate genes and to try with other suitable hosts are required.

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

PENGKLONAN DAN PENYATAAN GEN FIMBRIA DARI *PASTEURELLA MULTOCIDA* TIP 6:B, DIPENCILKAN DARIPADA LEMBU DENGAN HAWAR BERDARAH

Oleh

ERNIE ZURAIDA BINTI ALI

Jun 2005

Pengerusi: **Profesor Mohd. Zamri Saad, PhD**

Fakulti: **Perubatan Veterinar**

Hawar berdarah merupakan penyakit yang biasanya menyerang lembu dan kerbau, khususnya di Asia. Di Malaysia, strain *Pasteurella multocida* serotip 6:B lazimnya dipencilkan daripada kawasan yang terdapat penyakit hawar berdarah. Oleh itu, kebanyakkan kandungan keantigenan *P. multocida* telah dikaji seperti lipopolisakarida (LPS), protin selaput luar (OMP) dan kapsul. Walaubagaimanapun, fimbria yang terlibat dalam perlekatan pada permukaan sel perumah dan selalunya terlibat dengan kevirulenan organisma tidak dikaji. Oleh itu, kajian ke atas gen dan protin fimbria mungkin berguna dalam penghasilan vaksin bagi melindungi hawar berdarah.

Dalam kajian ini, gen fimbria *P. multocida* serotip B telah diamplifikasi, diklon dan digunakan untuk penjujukan DNA, dan diekspreskan ke dalam sistem penyataan *Pseudomonas aeruginosa* dan *Escherichia coli*. Kesemua isolat menghasilkan satu jaluran dengan berat molekul lebih kurang 450 bp. Analisis jujukan gen fimbria strain tip 6:B telah dibandingkan dengan *P. multocida* strain tip A:1 dan A:3. Jujukan

dari strain A:3 dan 6:B menunjukkan persamaan yang lengkap sementara jujukan dari strain A:1 dan 6:B menunjukkan 81.8 % persamaan asid amino. Walaupun *P. multocida* dan spesies yang lain berkongsi iaitu menunjukkan persamaan pada bahagian kematangan fimbria, kedua-duanya memperlihatkan perbezaan pada isyarat peptida, walaupun di dalam kumpulan yang sama.

Pasteurella multocida fimbria telah diklon dalam vektor penyataan pUCpKS/SK dan pCRT7®-TOPO bagi membina plasmid rekombinan. Di dalam SDS-PAGE, dapat di lihat bahawa sel rekombinan *P. aeruginosa* gagal untuk menghasilkan fimbria walaupun menggunakan kaedah permukaan fimbria yang spesifik. Analisis sap Western menggunakan antisera anti- fimbria *P. aeruginosa*, mendapati tindakbalas berlaku dengan *Pseudomonas* asal dan dengan keseluruhan rekombinan sel *P. aeruginosa*. Walaubagaimanapun, hanya *P. aeruginosa* asal menunjukkan tindakbalas silang apabila dititikkan atau diserapkan dengan antisera anti- fimbria *P. multocida*. Ini menunjukkan bahawa *P. aeruginosa* asal berkongsi epitop yang sama dengan *P. multocida* dan fimbria *P. multocida* tidak boleh diekspres di dalam *P. aeruginosa*.

Dalam sel *E. coli*, protin rekombinan telah dinyatakan sebagai protin larut tetapi pada aras agak rendah walaupun telah dioptimumkan. Dalam analisi sap Western menggunakan antibodi poliklon anti-*P. multocida* fimbria antisera, rekombinan protin telah dikenalpasti sebagai jaluran protin yang berat molekul lebih kurang 18 kDa. Walaubagaimanapun, ia tidak diketahui sama ada fimbria asal tidak diekspreskan atau gen tersebut diekspreskan tetapi tidak dapat dikeluarkan daripada

sel tersebut. Dengan demikian, analisis lanjutan diperlukan untuk mengenalpasti gen yang lain atau mencuba dengan vektor yang lain.

ACKNOWLEDGEMENTS

All praise to Almighty Allah, the Merciful and Benevolent. The completion of this study would not have been possible had it not been due to His will and favor.

I would like to express my sincere gratitude and appreciation to my supervisor Prof. Dr. Mohd. Zamri Saad for his invaluable guidance, advice, supervision and encouragement which has been a great favor on my behalf.

My sincere gratitude and appreciation to Assoc. Prof. Dr. Abdul Rahman Omar and Dr. Zunita Zakaria, who are my co-supervisors for their continuous guidance, suggestion, supports and in offering insightful suggestions towards the completion of this study.

Special thanks and sincere appreciation are due to Dr. Md. Sabri Mohd Yusoff and Dr. Siti Khairani Bejo, for the resourceful comments and suggestion during the completion of the bench works. I also like to express my thanks and gratitude to the staff of Histopathology Laboratory, Mr. Mohd. Jamil Samad, Bacteriology Laboratory, Mr. Mohd. Jefri Norsidin, Mr. Mohd. Hajaraih Selamat and Miss Latifah Hanan, Biologic Laboratory, Mrs. Rodiah Husin for their valuable technical assistance and those who contributed directly or indirectly in sharing their knowledge, skill and assistance throughout the course of my study.

I have also been very fortunate in receiving assistance and support from members of my colleagues and friends. I wish to express my sincere gratitude to Miss Shaherny

Zaid, Mr. Zulkefley Othman, Mr. Wan Keng Fei, Miss Balkis Abd Latip, Miss Norbazlin Marham, Mrs. Fariza Zainul Abidin and Miss Junainah Asli for their support in materializing this study.

Last but not least, I would like to express my deepest gratitude and thanks to my beloved parents, Tuan Hj. Ali Alias and Puan Hjh. Salnah Ali, my sister, Ernie Suzana Ali, dearest sisters and brothers for their endless support and trust. For Mohd Amran Mohd Radzi, thanks for your encouragement, patience and understanding, which had helped me complete this research study.

TABLE OF CONTENTS

LIST OF TABLE

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percentage
°C	Celsius temperature (centigrade temperature)
µg	Microgram
µl	Microliter
APS	Ammonium persulfate
Bp	Basepairs
BSA	Bovine serum albumin
cfu	colony forming unit
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene-diamine-tetraacetic acid
g	gram
H_2O	Water
i.e	In example
IPTG	Isopropyl-β-D-thiogalacosidase
Kb	Kilobase pair
kDa	Kilodalton
LB	Luria-bertani
L	liter
M	Molar
mg	milligram
$MgCl_2$	Magnesium chloride
ml	mililiter
mM	Milimolar

OD	Optical density
ORF	Open reading frame
Na_2HPO_4	di-sodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO_4	Sodium di-hydrogen phosphate
NaOH	Sodium hydrogen peroxide
nm	nanometer
PBS	Phosphate buffer saline
pH	puissance hydrogen (Hydrogen-ion concentration)
pPMTB3	Recombinant plasmid (pCR®2.1+fimbrial gene of *P. multocida*)
pRFSK	Recombinant plasmid (pUCpSK+fimbrial gene of *P. multocida*)
ptfA	Fimbrial gene of *Pasteurella multocida* A:1
RFSKP	Recombinant protein (fimbrial protein of *P. multocida* was expressed in *P. aeruginosa*
RSK	Control of recombinant protein (pUCpSK only)
rpm	round per minute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBE	Tris-Base-EDTA-buffer
TBS	Tris-buffer saline
TEN	Tris-EDTA-NaCl buffer
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Haemorrhagic septicaemia (HS) is an economically important disease of cattle and buffaloes in South East Asia (Rishendra and Jaiswal, 1998). The disease was first reported in Malaysia as early as the 1880's (FAO 1993) (Chandrasekaran, 1993 and De Alwis, 1999). The outbreaks of this disease are recorded regularly in many countries and account for heavily tool of cattle and buffaloes every year (Carter, 1974; Bain *et al.*, 1982; Carter *et al.*, 1987; Giridher *et al.*, 1990). In 1957, Bain estimated that the annual loss due to HS in Asia alone exceeded 100,000 susceptible animals (Josephs, 1979). During 1990-1999, the losses due to HS were estimated at RM 2.25 million (De Alwis, 1999). In Malaysia, the ruminant production systems are gradually changing from subsistence to intensive operations (Jamaluddin, 1992). The disease causes serious losses due to death, condemnation losses and costs of vaccination and medication.

Haemorrhagic septicaemia can be caused by one of two serotypes of *P. multocida* designated 6:B and 6:E (Namioka-Carter system) or B2 and E2 (Carter- Heddleston system) (De Alwis, 1990). *Pasteurella multocida* is also associated with a wide range of diseases, including fowl cholera of poultry and wild fowl, atrophic rhinitis of swine, haemorrhagic septicaemia of cattle and buffaloes and snuffles in rabbit. This organism can also cause diseases in humans such as sinusitis and its infection normally involves animal contact (Ruffolo *et al.*, 1997). *Pasteurella multocida* is a

Gram-negative, facultative anaerobe and non-sporogenous (Rimler and Rhoades, 1989).

Vaccination is the principal method of controlling HS in many countries (Carter, 1973; Bain *et al.,* 1982; Carter *et al.*, 1987; Giridhar *et al.*, 1990). Vaccines commonly used in this country are the alum-precipitated vaccine (APV) and the oil adjuvant vaccine (OAV). The APV is recommended for the in-contact animals in the area of an outbreak while the OAV is used for prophylaxis and is the most potent of the available vaccines (Carter and De Alwis, 1989). Although considerable reduction in deaths has been achieved by immunisation with the currently available vaccines, problems of HS outbreaks and deaths remain. Some of the most common problems are the low coverage of vaccination, occasional breakdown in the immunity in areas covered by vaccination and vaccines in low dosage, composition, quality and efficacy (Dawkins *et al.*, 1990). In order to overcome the problem, there is a need to improve the quality and effectiveness of the vaccines.

Several antigenic components of *P. multocida* have been investigated, which include the LPS (Rhoades and Rimler, 1991), LPS-protein complex (Tsuji and Matsumoto, 1988) and the outer membrane protein. The OMP of *P. multocida* have been extensively studied as potential vaccine candidates (Lutenberg *et al.*, 1986; Rimler and Rhoades, 1989; Lu *et al.*, 1991a, b; Manoha *et al.*, 1994, Ruffolo and Adler, 1996) but the outcome was inconclusive (Zamirah., 2002).

Fimbriae have been observed in a few strains of *P. multocida.* Fimbriae can enhance colonisation and attachment to the host cell surface, and is usually correlated with

virulence (Heckels *et al.*, 1989; Virji *et al.*, 1993). Therefore, investigation on the role of fimbriae can be beneficial and may be essential for vaccine development as observed against ovine footroot and bovine keratoconjunctivitis (Adler *et al.*, 1999).

To date, few studies had been carried out on the characterisation of *P. multocida* 6:B fimbriae. Therefore, the objectives of this study were:

1. to amplify, clone and sequence the fimbriae subunit gene of *P. multocida* serotype 6:B.
2. to express the fimbrial subunit gene of *P. multocida* 6:B in *P. aeruginosa.*
3. to express the fimbrial subunit gene of *P. multocida* 6:B in *E. coli.*

CHAPTER 2

LITERATURE REVIEW

2.1 Haemorrhagic septicaemia

Haemorrhagic septicaemia is a disease that occurs in Southern Europe Africa, Near and Middle East countries and throughout South East Asia (Joseph, 1979; Bain *et al.*, 1982; De Alwis 1999). Haemorrhagic septicaemia occurs in outbreaks during periods of environmental stress. During the intervening periods, the causative organism persists in the tonsil and nasopharyngeal regions and such animals serve as carriers of the disease (Mustafa *et al.*, 1978; Hiramure and De Alwis *et al.*, 1990).

The disease is most commonly observed in cattle and buffaloes, caused by two specific serotypes of the bacterium; *Pasteurella multocida* (Carter, 1973; Joseph, 1979; Bain *et al.*, 1982; Townsend *et al.*, 1996). Generally, the observed signs are elevated temperature, loss of appetite, nasal discharge, salivation and labored breathing with swelling in the submandibular region. It is an acute, fatal disease and one of the most economically important diseases of livestock (Dawkins *et al.*, 1990).

Haemorrhagic septicaemia caused a great economic loss in Asia, where buffaloes were reported to be particularly susceptible (Bain *et al.*, 1982; De Alwis 1999). In Malaysia, the mortality rate due to haemorrhagic septicaemia is higher in buffaloes than cattle (Joseph, 1979). It was found that poor husbandry practices and disease surveillance system cause the many outbreaks in this region (De Alwis, 1999).