



UNIVERSITI PUTRA MALAYSIA

**MOLECULAR CHARACTERISATION AND PATHOGENICITY
OF *PASTEURELLA MULTOCIDA* ISOLATED IN CHICKENS WITH
FOWL CHOLERA**

ANUN BINTI MAN

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FOWL CHOLERA**

By

ANUN BINTI MAN

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

July 2001



DEDICATION

**WITH APPRECIATION AND RESPECT,
THIS THESIS IS DEDICATED**

TO

MY MOTHER AND MY FAMILY WHO INSPIRED ME

AND

MAKE IT ALL WORTHWHILE



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

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OF *PASTEURELLA MULTOCIDA* ISOLATED IN CHICKENS WITH
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Chairman : Associate Professor Mohd Zamri Saad, DVM, Ph.D.

Faculty : Veterinary Medicine

Twenty-two isolates of *Pasteurella multocida* isolated from outbreaks of fowl cholera in chickens were studied. The isolates identified as *Pasteurella multocida* serotypes A:1, A:3 and A:1,3 based on capsular and somatic serotyping results were analysed for their DNA and protein profiles, pathogenicity and antigenicity. Genomic DNA analysis using several endonuclease enzymes by restriction endonuclease analysis (REA) and random amplified polymorphic DNA (RAPD) analysis revealed generally similar DNA profiles for all 22 isolates of *Pasteurella multocida*, except minor differences as detected upon digestion with *Hha*I. Molecular characterisation in the form of REA and RAPD in this study was extremely subtle to differentiate between strains of *Pasteurella multocida* and need further clarification along with the used of these techniques.



Among endonuclease enzymes used, *HhaI* was the only useful enzyme for differentiating strains of *Pasteurella multocida*. The DNA profiles obtained reflected well in serotyping and pathogenicity. This may suggest that the genetic elements encode certain virulence factors of *Pasteurella multocida* strains. However, there is no conclusive evidence for the role of plasmids and outer membrane protein (OMP) in the pathogenicity of *Pasteurella multocida* strains. All isolates that contain no plasmids and the similar OMP profiles produced difference pathogenicity among serotypes. Pathogenicity study revealed that serotype A:1,3 is more pathogenic than serotypes A:1 and A:3. The rapid death and pathological changes in chicken died shortly following inoculation with pathogenic strain were considered to be indicative of shock and attributed to the action of endotoxin.

The OMP analysis using SDS-PAGE revealed that all serotypes contained similar size of protein in the range of 29.0 – 107.0 kDa. A single major protein of 36.0 kDa, believed to be 'H' protein/porin of *Pasteurella multocida* was identified in all serotypes. However, there was no direct evidence to associate this protein with pathogenicity. The degree of OMP antigenic sharing among serotypes is extensive as revealed in immunoblotting. The 36.0 kDa protein was the most serologically major reactive antigenic protein and recognised by both homologous and heterologous antisera. Almost all antigenic proteins of serotype A:1 were expressed in both serotypes A:3 and A:1,3. Results obtained may suggest that OMP of *Pasteurella multocida* serotype A:1 could be the potential vaccine candidate as it may share antigens that are immunologically protective.



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sebagai memenuhi keperluan untuk Ijazah Master Sains Veterinar

**PENCIRIAN MOLEKUL DAN KEPATOGENAN
PASTEURELLA MULTOCIDA YANG DIPENCILKAN DARIPADA AYAM
BERPENYAKIT KOLERA**

Oleh

ANUN BINTI MAN

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Dua puluh dua isolat *Pasteurella multocida* yang dipencilkan daripada wabak penyakit kolera ayam telah dikaji. Isolat yang telah dikenalpasti sebagai *Pasteurella multocida* serotip A:1, A:3 dan A:1,3 berdasarkan keputusan ujian pengkelasan kapsul dan soma, telah dianalisis untuk profil DNA dan protin, kepatogenan dan keantigenan. Analisis genom DNA dengan menggunakan beberapa enzim endonuklease melalui analisis endonuklease tersekat (REA) dan amplikasi rawak polimorfisme (RAPD) menghasilkan profil DNA yang pada keseluruhannya sama bagi semua 22 isolat *Pasteurella multocida*, kecuali perbezaan kecil diperolehi apabila *HhaI* digunakan. Dalam kajian ini, pencirian molekul secara REA dan RAPD memberikan perbezaan yang tidak ketara dalam membezakan strain *Pasteurella multocida* dan penjelasan yang selanjutnya diperlukan melalui penggunaan teknik-teknik ini.



Di antara enzim endonuklease yang telah digunakan, *HhaI* merupakan enzim yang boleh membezakan strain *Pasteurella multocida*. Profil DNA yang terhasil didapati berkait rapat dengan serotip dan kepatogenan. Adalah dicadangkan bahawa elemen genetik mengkodkan sesetengah faktor kepatogenan strain *Pasteurella multocida*. Walau bagaimanapun, kajian ini tidak menunjukkan keterangan yang kukuh, tentang peranan plasmid dan protin selaput luar (OMP) dalam kepatogenan strain *Pasteurella multocida*. Semua isolat tidak mengandungi plasmid dan sama profil OMP menghasilkan kepatogenan yang berbeza di antara serotip. Kajian kepatogenan mendapati bahawa *Pasteurella multocida* serotip A:1,3 lebih patogen berbanding serotip A:1 dan A:3. Kematian yang cepat dan perubahan patologi yang berlaku pada ayam sebaik sahaja diinokulkan dengan strain yang patogen dipertimbangkan sebagai penandaan kejutan dan berpunca daripada tindakan endotoksin.

Analisis OMP menggunakan sodium dodecil sulfat-gel elektroforesis poliakrilamida (SDS-PAGE) menunjukkan bahawa semua serotip mengandungi protin yang sama saiznya dengan purata 29.0 – 107.0 kDa. Satu protin utama pada 36.0 kDa, yang dipercayai protin 'H'/porin *Pasteurella multocida* telah dikenalpasti pada semua serotip. Walau bagaimanapun, tiada keterangan secara langsung yang boleh mengaitkan protin ini dengan kepatogenan. Sebagaimana yang diperlihatkan dalam pembloktan imun, darjah perkongsian antigen OMP di antara serotip adalah ketara. Protin 36.0 kDa adalah protin antigen yang paling reaktif, dan boleh dikenalpasti oleh kedua-dua antiserum homologous dan heterologous. Hampir semua

protin antigen pada serotip A:1 telah ditunjukkan dalam kedua-dua serotip A:3 dan A:1,3. Keputusan yang diperolehi menggambarkan bahawa OMP *Pasteurella multocida* serotip A:1 berpotensi sebagai calon vaksin kerana ianya mungkin berkongsi antigen yang boleh melindungi secara imunologi.



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LIST OF ABBREVIATIONS

BHI	Brain heart infusion
BSA	Bovine serum albumin
CAM	Chorio-allantoic membrane
Cfu	Colony forming units (bacteria)
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Kbp	Kilobase pairs
KDa	Kilodalton
LPS	Lipopolysaccharide
M	Molar
MW	Molecular weight
OMP	Outer membrane protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PH	Hydrogen ion concentration
pi	Post-inoculation
RAPD	Random amplified polymorphic DNA analysis
REA	Restriction endonuclease analysis
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TBE	Tris-borate EDTA
V	Volt
v/v	Volume to volume ratio
w/v	Weight to volume ratio



CHAPTER 1

INTRODUCTION

The animal pathogen *Pasteurella multocida* is 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 rabbits. The disease causes serious losses due to deaths, condemnation losses, and vaccination and medication costs. In poultry, fowl cholera is a highly contagious disease, which results in significant economic losses to the poultry industry worldwide (Hird *et al.*, 1991; Rhoades and Rimler, 1991). Typically, fowl cholera occurs abruptly in healthy turkeys of market age; it causes high mortality over a short time and/or high condemnation rates at processing plants, despite vigorous medication efforts. Heavy losses have been observed in mature chickens for up to 16 week of age. This means that mature chickens are more susceptible than young chickens (Heddleston, 1962; Hungerford, 1968). The severity of the disease and its incidence are also influenced by environment factors such as overcrowding, climatic changes, nutrition and concurrent diseases (Alberts and Graham, 1948).

Many birds and mammals can be infected with *Pasteurella multocida* without serious consequences (Snipes *et al.*, 1988a). In turn, they serve as the source of infection when they come into contact with poultry. Therefore, preventive efforts should include complete isolation of poultry flocks and strict sanitation combined with restricted traffic to and from poultry houses.



Fowl cholera can result from infection with strains of *Pasteurella multocida* representing the variation in somatic serotypes and capsular serogroup (Rhoades and Rimler, 1991; Snipes *et al.*, 1990). Currently, 16 serotypes (serotypes 1-16) and four capsular serogroups (A, B, D and F) are represented among strains isolated from avian hosts (Hofacre and Glisson, 1986; Rhoades and Rimler, 1987; Rimler and Rhoades, 1987). Although several serotypes of *Pasteurella multocida* within serogroup A are regarded as the primary cause of fowl cholera (Rhoades and Rimler, 1987; Rimler and Rhoades, 1989b) and serotypes A:1, A:3 and A:3,4 apparently dominate among the strains (Cutis, 1981; Rhoades and Rimler, 1990b), there were no indications that any particular serotype is more or less virulent than others. In fact, different isolates of the common serotype A:3,4 have been shown to vary greatly in virulence (Lee *et al.*, 1988). Thus, somatic serotyping seems of little use in assessing the virulence of strains of *Pasteurella multocida*.

In recent years, identification and characterisation has favoured analyses that reflect one of the most fundamental properties of an organism, its genetic information. The use of molecular characterisation in the form of restriction endonuclease analysis (REA) and random amplified polymorphic DNA analysis (RAPD), have been applied to identify and to differentiate *Pasteurella multocida* strains (Zucher *et al.*, 1996). The ability to differentiate phenotypically similar isolates is critically important in epidemiology, particularly when establishing the identity of bacterial vaccine strains (Stull *et al.*, 1988). Further study in correlation of certain serotype with lesions will help to understand detail on pathogenicity and

virulence factors of the *Pasteurella multocida*. Several factors that may be important for the virulence of *Pasteurella multocida* are lipopolysaccharide (LPS) (Rimler *et al.*, 1984), capsule (Tsuji and Matsumoto, 1989), plasmids and resistance to complement-mediated bacteriolysis (Lee *et al.*, 1991). Studies have been undertaken to determine the presence of plasmids in numerous strains of *Pasteurella multocida* and to investigate the correlation between antibiotic resistance profiles (Hirsh *et al.*, 1985; 1989), virulence attributes (Lee and Wooley, 1995) and the presence of plasmids. The plasmids have been speculated to encode the virulence factors involved. Avian strains of *Pasteurella multocida* have been shown to harbour plasmids (Snipes *et al.*, 1990; Price *et al.*, 1993; Diallo *et al.*, 1995) but the rate of plasmid carriage has been shown to vary considerably between isolates from 24% (Price *et al.*, 1993) to 70.7% (Hirsh *et al.*, 1989).

Several antigenic components have been investigated as an immunogen against *Pasteurella multocida* infection including purified lipopolysaccharide (LPS) (Rhoades and Rimler, 1991) and LPS-protein complex (Tsuji and Matsumoto, 1988b). However, the role of LPS as an immunogen remains controversial particularly in mammals. Mice, cattle and rabbits have not been readily protected against *Pasteurella multocida* infection following immunisation with LPS (Rimler and Rhoades, 1989c). Recently, outer membrane proteins (OMPs) of *Pasteurella multocida* have been studied as potential immunogens, which make them potential vaccine candidates (Lugtenberg *et al.*, 1986; Rimler and Rhoades, 1989c; Lu *et al.*, 1991a,b,c; Manoha *et al.*, 1994; Ruffolo and Adler, 1996). OMPs of avian origin of

