UNIVERSITI PUTRA MALAYSIA

PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* OBTAINED FROM POULTRY IN IRAN

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By

AHMAD REZA JABBARI

Thesis Submitted in Fulfilment of the Requirement for the Degree of
Doctor of Philosophy in the Faculty of Veterinary Medicine
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September 2001

DEDICATED TO MY WIFE, MAHTAB MOZAFFARI,

OUR TWO SONS, MOHAMMAD VAHID

AND MOHAMMAD MOEIN

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

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September 2001

Chairman: Associate Professor Dr. Abdul Aziz Saharee

Faculty: Veterinary Medicine

A collection of twenty five *Pasteurella multocida* isolates obtained from avian pasteurellosis in northern part (endemic area) of Iran were studied for some of their phenotypic and molecular characteristics. This research is the first study on conducting of serotyping and molecular characterization of avian *P.multocida* in Iran.

Based on the biochemical characteristics, all *P.multocida* isolates tested belonged to subspecies (biotype) multocida. Antimicrobial sensitivity test showed that all the isolates examined were resistant to at least three of the thirteen antimicrobials tested. Among the antimicrobial agents, chloramphenicol, combination of sulfametoxazin and trimetoprim and nitrofurantoin were found to be the most effective (100% sensitivity)

followed by tetracycline (96% sensitivity), penicillin (88% sensitivity) and gentamycin (76% sensitivity). The highest percentage of resistance was found against lincomycin, bacitracin and cloxacillin (100% resistant) followed by furazolidone and colistin (84% and 68 % respectively).

Agar gel diffusion precipitation (AGDP) test was used to determine somatic serotypes of the isolates. According to the results of the AGDP test, Serotype 1 was dominant among avian isolates from endemic area. Serotypes 3, 3 × 4 and 4, found for the first time in the country were also identified among the isolates.

Electrophoresis protein patterns of the isolates were studied by using sodium dodecyl sulphate polyacylamide gel (SDS-PAGE). All strains were similar in the majority of protein bands. The main difference between protein patterns of the isolates was revealed in the position of one of the major band (H Protein) presented in the 34- 38 KDa region. According to H protein position, three distinguishable groups were identified. In protein type I, the molecular mass of H protein was about 38 KDa but in protein types II and III it was 36.5 and 34 KDa respectively.

The minimum lethal dose (MLD) of the strains with protein types I, II and III as a virulence determinant was identified in mice. It was revealed that the strain with protein type III had the least virulence and the strain with protein

type I had the greatest virulence in mice. Immunization of mice with strain PMI030 (protein type I) induced a good protection against homologous protein type challenge.

Restriction enzyme analysis (REA) of chromosomal DNA and repetitive extragenic palindromic elements PCR (REP-PCR) were used for determination of genetic diversity among the isolates. DNA fingerprinting by HpaII digestion divided the twenty-five isolates into 7 REA groups, 2 of which contained a single isolate.

DNA fingerprinting with REP-PCR revealed a great genetic diversity among the isolates. According to amplified DNA patterns, a total of 9 REP-PCR groups were determined. REP-PCR produced amplified bands ranging in size from approximately 700 bp to 3.6 Kb with two species-specific bands of 0.8 Kb and 2.3 Kb. REP-PCR was able to differentiate *P.multocida* isolates from different source and geographical areas. Results of this study showed that the use of REP element amplification by polymerase chain reaction is highly reproducible and can be suggested as a suitable epidemiologic tool especially for investigation on the origin of outbreaks and similarity between different avian isolates of *P.multocida*.

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

CIRI-CIRI FENOTIPIK DAN MOLEKULAR *PASTEURELA MULTOCIDA* YANG DIPEROLEHI DARIPADA TERNAKAN AYAM ITIK DI IRAN

Oleh

AHMAD REZA JABBARI

September 2001

Pengerusi: Profesor Madya Dr. Abdul Aziz Saharee

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Satu kajian tentang sekumpulan 25 *Pasteurella multocida* yang diperolehi daripada pasteurelosis avian di bahagian utara (kawasan endemi) Iran telah dijalankan untuk meneliti ciri-ciri fenotipik dan molekularnya. Kajian ini merupakan yang pertama mengkaji tentang kelakuan serotip dan ciri-ciri molekular *P.multocida* avian di Iran.

Berasaskan ciri-ciri biokimia, kesemua pemencilan *P.multocida* yang diuji tergolong sebagai subspesies (biotaip) multocida. Ujian sensitiviti antimikrob menunjukkan bahawa kesemua pemencilan yang diteliti menolak sekurang-kurangnya 3 daripada 13 antimikrob yang diuji. Di kalangan agen-agen antimikrob, kloramfenikol, kombinasi sulfametoksazin dan trimetoprim serta nitrofurantin didapati paling efektif (sensitiviti 100%) diikuti oleh tetrasiklin

(sensitiviti 98%), penisilin (sensitiviti 88%) dan gentamisin (sensitiviti 76%). Peratus tertinggi resistan didapati lebih kepada linkomisin, basitrasin dan kloksasilin (resistan 100%), diikuti oleh furazolidon dan kolistin (masing-masing 84% dan 68%).

Ujian Agar Gel Diffusion Precipitation (AGDP) telah digunakan untuk menentukan pemencilan somatik serotip. Berdasarkan keputusan ujian AGDP, Serotip 1 didapati dominan berbanding avian yang dipencilkan daripada kawasan endemik. Serotip 3, 3 x 4 dan 4 yang pertama kali ditemui di negara Iran juga dikenal pasti di kalangan pemencilan-pemencilan lain.

Corak protein elektroforisis bagi pemencilan ini telah dikaji dengan menggunakan gel sodium dodisil sulfat poliklamid (SDS-PAGE). Kesemua strain adalah sama dalam kebanyakan jalur-jalur protein. Perbezaan utama antara corak-corak protein pemencilan ini begitu ketara pada kedudukan salah satu daripada jalur utama (H Protein) yang dibentangkan dalam kawasan 34-38 Kda. Merujuk kepada kedudukan protein H, tiga kumpulan yang berbeza telah dikenal pasti. Pada protein jenis I, jisim molekular protein H adalah lebih kurang 38 Kda tetapi di dalam protein jenis II dan jenis III, masing-masing 36.5 dan 34 Kda.

Dos lethal minimum (MLD) strain berprotein jenis I, II dan III sebagai penentu virulen telah dikenal pasti pada tikus. Ia menunjukkan bahawa strain berprotein jenis III mempunyai virulen paling sedikit dan strain berprotein jenis I mempunyai virulen paling banyak terdapat pada tikus.

Pembatasan analisis enzim (REA) kromosomal DNA dan REP-PCR digunakan untuk menentukan kepelbagaian genetik di kalangan pemencilan. Cap jari DNA oleh pencernaan HpaII membahagikan 25 pemencilan ini kepada 7 kumpulan REA, yang mana dua daripadanya mengandungi pemencilan tunggal.

Cap jari DNA beserta REP-PCR menunjukkan kepelbagaian genetik yang amat ketara di kalangan pemencilan. Berdasarkan corak DNA yang luas, sejumlah 9 REP-PCR telah ditentukan. REP-PCR menghasilkan jalur-jalur yang kuat, selari mengikut saiz daripada kira-kira 700 bp hingga 3.6 Kb beserta dua jalur spesifik-spesies 0.8 Kb dan 2.3 Kb. REP-PCR berupaya untuk membezakan pemencilan *P.multocida* di kalangan sumber-sumber dan kawasan-kawasan geografi yang berbeza. Kesimpulan daripada kajian ini menunjukkan bahawa penggunaan amplifikasi elemen-elemen REP oleh tindak balas berantai polimeras sangat produktif dan boleh dicadangkan sebagai alat epidemiologi yang sesuai untuk penyelidikan terhadap kerebakan asal dan keserupaan antara pemencilan avian *P.multocida* yang berlainan.

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I certify that Examination Committee met on 20th September 2001 to conduct the final examination of Ahmad Reza Jabbari on his Doctor of Philosophy thesis entitled "Phenotypic and Molecular Characterization of *Pasteurella multocida* Obtained from Poultry in Iran" in accordance with 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:

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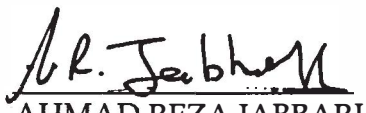
I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at UPM or other institutions.



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LIST OF ABBREVIATIONS

AGDP	Agar Gel Diffusion Precipitin Test
AP-PCR	Arbitrary Primed PCR
APS	Ammonium Persulfate
BHI	Brain Heart Infusion
°C	Degree Celcius
CCA	Crude Capsule Antigens
CCF	Cell-Free Culture Filtrate
CFU	Colony Forming Unit
CP	Capsular Polysaccharide
CPFs	Cross-Protection Factors
CU strain	Clemson University Strain
DATP	Deoxyadenosine Triphosphate
DCTP	Deoxycytidine Triphosphate
DGTP	Deoxyguanosine Triphosphate
DTTP	Deoxythymidine Triphosphate
DNTP	Deoxynucleutide Triphosphate
DIE	Descriptive Identification Epithet
EDTA	Ethylendiamine Tetra Acetate
ELISA	Enzyme Linked Immunosorbent Assay
FC	Fowl Cholera
HS	Haemorrhagic Septicaemia
HSA	Heat Stable Antigen
HSPs	Heat Shock Proteins
IHA	Indirect Haemaglutination Test
IVO	Iranian Veterinary Organization
KDa	Kilo Daltons
KSCN	Potassium Thiocyanate Extracts
LD 100	Lethal Dose of 100 Percent
LPS	Lipopolysaccharide
MAb	Monoclonal Antibody
Mda	Mega Daltons
MHA	Muller-Hinton Agar
MLD	Minimum Lethal Dose
MOMPs	Major Outer Membrane Proteins
OMPs	Outer Memberane Proteins
P.multocida	*Pasteurella multocida*
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PMSF	Phenylmethyl Sulfonyl Fluoride
PMT	*Pasteurella multocida* Toxin

RAPD	Random Amplified Polymorphic DNA
RD Complex	Respiratory Disease Complex
REA	Restriction Endonuclease Analysis
REP	Repetitive Extragenic Palindrome
rRNA	Ribosomal RNA
RVSRI	Razi Vaccine and Serum Research Institute
Taq	*Termophilus aquaticus*
SDS	Sodium Dodecyl sulphate
SPF	Specific Pathogen Free
Subsp	Subspecies
TAE	Tris Acetate EDTA
TEMED	Tetrametylendiamine
Tox	Toxin
TSI	Three Sugar Iron Agar
U	Unit
VRI	Veterinary Research Institute
WC	Whole Cell