UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY OF Pasteurella multocida ISOLATED FROM COMMERCIAL POULTRY FARM AND FREE-FLYING BIRDS

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

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

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November 2019

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Pasteurella multocida has been recognized as the causative agent of various infections in a wide range of mammals and bird species. *Pasteurella multocida* could lead to significant losses in both poultry and wild birds. In poultry, *P. multocida* can cause acute septicemic disease with high morbidity and mortality; however, localized chronic infection is more often reported. *Pasteurella multocida* can be classified into five serogroups (A, B, D, E, and F) based on the capsular antigen. The first objective of this study is to isolate, identify, and serotype *P. multocida* from poultry farms in the central region of Peninsular Malaysia. A total of 372 samples, consisting of oral swabs and internal organs of chickens from 31 poultry farms, and 59 oral swabs from free-flying birds were collected in the central region of Peninsular Malaysia. *P. multocida* was identified using biochemical test and polymerase chain reaction (PCR). Thirty-four samples from five chicken farms were positive for *P. multocida*, while none of the free-flying birds contained *P. multocida*. All isolates were identified as serotype A, except for one isolate as serotype F using the PCR method. The second objective is to determine the antimicrobial susceptibility profile using disc diffusion method among *P. multocida* isolates. Test against commonly used antibiotics in poultry showed that these isolates showed high rate of resistance against erythromycin (100%), moderate rate of resistance against streptomycin (80%), enrofloxacin (80%), and susceptible to penicillin G (100%), amoxicillin (100%), gentamicin (97%), florfenicol (97%), and tetracycline (94%). The third objective is to molecularly characterize *P. multocida* isolates using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Pulsed-field gel electrophoresis results showed that 31 isolates were typable, with four distinct profiles evident among the isolates. Multi Locus Sequence Typing typing was conducted on one isolate per farm and the untypeable PFGE isolates. Seven different sequence types (ST) were identified, namely ST8, ST200, ST157, ST214, ST354, ST356, and ST357. Of these, ST354, ST356, and ST357 were identified for the first time. The fourth objective is to characterize *P. multocida* isolated from chicken farms in the central region of Peninsular Malaysia in between year 2000 to 2018. Thirteen isolates of *P. multocida*

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from samples submitted to the bacteriology laboratory, Faculty of Veterinary Medicine, University Putra Malaysia, Malaysia, were characterized in this objective. These isolates showed several multidrug-resistant properties against antibiotics. Three different sequence types (ST) were identified among the laboratory isolates. The finding of the study provided additional epidemiological information on the strains of *P. multocida* that cause outbreaks of fowl cholera in the central region of Peninsular Malaysia. The results of this study contribute to the understanding of fowl cholera status in Malaysia with relations to the genetic diversity and antimicrobial resistance.

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PENCIRIAN MOLEKUL DAN KERENTANAN ANTIBIOTIK *Pasteurella multocida* YANG DIASINGKAN DARIPADA LADANG TERNAKAN KOMERSIAL DAN BURUNG TERBANG BEBAS

Oleh

SABSABI MOHAMMAD ATEIH

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Pasteurella multocida telah dikenali sebagai agen penyebab pelbagai jangkitan dalam pelbagai jenis mamalia dan juga spesies burung. *Pasteurella multocida* boleh mengakibatkan kerugian besar pada unggas dan burung liar. Pada unggas *P. multocida* boleh menyebabkan penyakit septikemik akut dengan morbiditi dan mortaliti yang tinggi, walau bagaimanapun, jangkitan kronik setempat lebih sering dilaporkan. *Pasteurella multocida* boleh dikelaskan kepada lima kumpulan sero (A, B, D, E, dan F) yang berdasarkan kepada antigen kapsul. Objektif pertama kajian ini adalah untuk memencil, kenalpasti, dan serotip *P. multocida* daripada ladang unggas di kawasan tengah semenanjung Malaysia. Dalam kajian ini, sebanyak 372 sampel terdiri daripada kikisan oral dan organ dalaman ayam dari 31 ladang ayam, dan 59 kikisan oral daripada burung yang terbang bebas telah dikumpulkan dari wilayah tengah Semenanjung Malaysia. *P. multocida* dikenalpasti menggunakan ujian biokimia dan reaksi rantai polimerase (PCR). Tiga puluh empat sampel dari 5 ladang ayam adalah positif untuk *P. multocida*, manakala burung yang terbang bebas adalah negatif untuk *P. multocida*. Semua asingan dikenalpasti sebagai serotip A, kecuali satu asingan adalah serotip F dengan menggunakan kaedah PCR. Objektif kedua adalah untuk menentukan profil kerentanan antimikrob menggunakan kaedah pembauran piring antara asingan *P. multocida*. Ujian kepekaan antibiotik menggunakan antibiotik umum dalam unggas menunjukkan bahawa asingan ini kadar ketahanan yang tinggi terhadap eritromisin (100%), kadar rintangan sederhana terhadap streptomisin (80%), enrofloxasin (80%), dan kadar mudah terdedah kepada penisilin G (100%), amoxysillin (100%), gentamisin 97%), florfenikol (97%), dan tetrasiklin (94%). Objektif ketiga adalah untuk mencirikan asingan *P. multocida* menggunakan 'pulsed-field gel electrophoresis (PFGE)' dan 'multilocus sequence typing (MLST)'. Keputusan PFGE menunjukkan bahawa 31 asingan telah dijeniskan dengan empat profil yang jelas. Penetapan MLST dilakukan pada satu asingan untuk setiap ladang dan juga pada asingan yang tidak dapat dijeniskan oleh PFGE. Tujuh jenis urutan yang berlainan telah dikenalpasti iaitu ST8, ST200, ST157, ST214, ST354, ST356, dan ST357. Daripada jumlah ini, ST354, ST356 dan

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ST357 telah dikenalpasti buat kali pertama. Objektif keempat adalah untuk mencirikan asingan *P. multocida* daripada ladang ayam di wilayah tengah semenanjung Malaysia di antara tahun 2000 hingga 2018. 13 asingan *P. multocida* dari kes-kes terdahulu yang dikemukakan kepada Makmal Bakteriologi juga direkrut dalam kajian ini. Asingan-asingan ini juga telah menunjukkan kerintangan terhadap pelbagai jenis antibiotik. Tiga jenis urutan yang berlainan telah dikenalpasti dalam kalangan asingan dari makmal. Penemuan kajian ini memberikan maklumat epidemiologi tambahan mengenai strain *P. multocida* yang menyebabkan wabak kolera ayam di wilayah tengah Semenanjung Malaysia. Hasil dari kajian ini menyumbang kepada pemahaman status kolera ayam di Malaysia berhubung dengan kepelbagaian genetik dan rintangan antimikrob.

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This Thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Veterinary Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µL	Micro litre
µM	Micro molar
ATCC	American Type Culture Collection
AST	Antibiotic susceptibility test
AMX	Amoxycillin
CHEF	Contour-clamped homogeneous electric field
CN	Gentamicin
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
ddH20	Double distilled water
E	Erythromycin
ENR	Enrofloxacin
FC	Fowl cholera
FFC	Florfenicol
Kb	Kilobase
MLST	Multilocus Sequence Typing
mPCR	Multiplex Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
P	Penicillin G
RAPD	Random amplified polymorphic DNA
RNase	Ribonuclease
ST	Sequence type

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S	Streptomycin
TE	Tetracycline
TBE	Tris-borate-EDTA
TE	Tris-EDTA
UV	Ultraviolet
UPGMA	Unweighted pair group arithmetic
UV	Ultraviolet
V	Volt
WHO	World Health Organization

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CHAPTER 1

INTRODUCTION

Pasteurella multocida is a significant Gram-negative pathogen that can cause acute respiratory infection in many species of animals, including bovine, swine, rabbits, and poultry (Harper et al., 2006). In poultry, the bacteria can cause fowl cholera (FC), which is characterized as a significant economic problem for poultry production, backyard poultry, and other free-flying birds (Rhoades and Rimler, 1991). Even though fowl cholera can cause acute septicemic infection with high mortality and morbidity up to 100%, chronic localized infection is often reported (Glisson et al., 2013). Outbreaks of fowl cholera were reported worldwide in many avian species, including wild birds in Amsterdam (Jaeger et al., 2018); waterfowl in Korai (Kwon and Kang, 2003); ducks, geese and chickens in China (Wang et al., 2013); chickens and ducks in India (Karthik et al., 2018); chickens in Indonesia (Jonas et al., 2001); chickens, goose, turkey, and wild birds in Japan (Nakamine et al., 1992); and backyard chickens in Australia (Singh et al., 2013).

Fowl cholera is also often reported in Malaysia. According to Arumugam et al. (2011), 28 isolates of avian *P. multocida* were recovered between 1996–2004 in Malaysia. Nafizah et al. (2014) reported that 18 isolates of *P. multocida* were retrieved from poultry from 2007 to 2010. Another study reported 15 isolates of avian *P. multocida* retrieved from 2014 to 2016 from samples submitted to Veterinary Research Institute, Ipoh, Malaysia (Khoo et al.2017).

Pasteurella multocida is morphologically characterized as a small Gram-negative, non-motile bacteria (Christensen and Bisgaard, 2006), and currently subdivided into four subspecies; namely subsp. *multocida*, subsp. *gallicida*, subsp. *septica*, and subsp. *tigris* (Harper et al., 2006). *Pasteurella multocida* can be further divided into five capsular serotypes A, B, D, E, and F. Four capsular serotypes A, B, D, and F were reported to cause infection in avian species. However, serotype A is the dominant serotype infecting avian species (Carter, 1955; Rhoades and Rimler, 1991; Wilkie et al., 2012). There are several methods of characterizing *Pasteurella multocida*. Capsular serotyping is a crucial step in characterizing the outbreak of fowl cholera. Townsend et al. (2001) optimized a multiplex PCR serotyping assay with high sensitivity rates for all *P. multocida* serotypes.

Antibiotics are significantly crucial for animal production, as it is being used as a feed supplement and as a treatment for diseases (Agyare et al. 2018). However, the excessive usage of antibiotics has increased the emergence of resistant bacterial strains. Currently, antibiotic resistance is a global problem due to the threat of treatment failure in the future and the increase of multidrug-resistant bacterial strains (Aarestrup et al., 2008). Several multidrug antibiotic resistance of avian *P. multocida* strains were reported for a wide range of antibiotics. A study by Jonas et al. (2001) reported that avian *P. multocida* isolates from Indonesia were found to be resistant to lincomycin and sulfadiazine while

another study by Balakrishnan and Roy (2012) shows that most of *P. multocida* isolates in India were found to be resistant to chloramphenicol and erythromycin.

Molecular typing method is a vital tool used in epidemiology studies to study the presence of different bacterial strains and to study the genetic evolution of the bacteria. Pulsed-field gel electrophoresis (PFGE), a widely used typing method, uses restriction enzyme to fingerprint bacterial chromosome. PFGE typing provides a high discriminating power among *P. multocida* strains (Gunawardana et al., 2000; Kardos and Kiss, 2005). Sthitmatee et al. (2010) used PFGE to study various strains of avian *P. multocida* in Japan and compared it to other isolates from the USA, Taiwan, and Indonesia and found relationship between the genetic profiles. Multilocus sequence typing (MLST) is another molecular-based typing method used to study the diversity of the sequence type (ST) and the evolution of bacterial strains. The main advantage of MLST is the ability to compare the presence of STs between different countries using a database, and currently, MLST is the gold standard for *P. multocida* typing (Subaaharan et al., 2010). Previous study showed that some of the STs were reported in nearby countries and suggested that the STs were transferred between the countries (Sarangi et al., 2016).

Poultry production is a significant food industry in Malaysia due to the high consumption of eggs and poultry meat. Although *P. multocida* outbreaks in poultry flocks were occasionally reported, there is a lack of information about the characterization of *P. multocida* in poultry. Besides, there is also a lack of data on antibiotic susceptibility profile of *P. multocida* isolates infecting birds in Malaysia. To date, very little data were published concerning the genetic diversity of avian *P. multocida* in Malaysia. Therefore, the aim of this study is to characterize *Pasteurella multocida* isolated from birds in Malaysia to further understand the genetic variation and antibiogram profiles of these isolates.

1.1 Study objectives

1) To isolate, identify, and serotype *P. multocida* from poultry farms in the central region of Peninsular Malaysia.
2) To determine the antimicrobial susceptibility profile among *P. multocida* isolates.
3) To molecularly characterize *P. multocida* isolates using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).
4) To characterize *P. multocida* isolated from chicken farms in the central region of Peninsular Malaysia in between year 2000 to 2018.

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1.2 Hypotheses

1) Majority of *Pasteurella multocida* isolates belongs to serotype A.
2) *Pasteurella multocida* isolates resistant to commonly used antibiotics in the poultry farms.
3) Close genetic relationship among *P. multocida* isolates from different farms.

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