UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF Mycoplasma gallisepticum AND Mycoplasma synoviae IN POULTRY AND BIRDS IN PENINSULAR MALAYSIA

TAIYARI HOSSEIN

FPV 2021 20

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By

TAIYARI HOSSEIN

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

June 2021

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

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June 2021

Chairman : Professor Jalila Abu, PhD
Faculty : Veterinary Medicine

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) continue to cause huge economic losses to poultry industries yearly. Acute and chronic respiratory disease (CRD), sinusitis, synovitis, massive loss of body weight, decreased egg production, and reduction of hatchability rate are the sequelae of avian mycoplasmosis. The emergence of mycoplasmal conjunctivitis in wild house finch populations emphasized mycoplasmas' natural aptitude in evolving adaptability to various avian hosts. This capability contributes to the emergence of new wild reservoirs, and ultimately, the circulation of pathogens in the environment. In Malaysia, apart from reports indicating high prevalence of MG infection among commercial and backyard poultry farms, a unique strain of MG has also been identified which highlights the importance of avian mycoplasmosis in Malaysia. Despite all these efforts, there is a lack of information on optimization of detection techniques (culture and PCR), fingerprinting strains isolated from different hosts, and finally, antibiotic susceptibility profile of the field isolates. Therefore, this study was carried out to isolate, molecular characterize, and determine the antimicrobial susceptibility of MG and MS isolates from poultry and non-poultry birds in Peninsular Malaysia. Before conducting sample collection, isolation optimization was conducted by comparing the efficacy of the commonly used mycoplasma media; Frey with swine serum (FMS) and modified PPLO (Chanock) in isolation of the organism. Results showed that FMS significantly increases the chance of isolation of MG and MS in comparison to Chanock medium. Therefore, FMS was used for isolation purpose. A total of 546 choanal slit swab samples were collected from different avian species and subjected to isolation and PCR. Using immunofluorescence assay (IFA), 36.3% (198/546) MG and MS isolates were detected of which 90.4% (179/198) isolates were from poultry, and 9.6% (19/198) isolates were from non-poultry birds. For non-poultry samples, 15.8% (3/19) samples had MG colonies, and 84.2% (16/19) samples had MS colonies. For poultry samples, 26.8% (48/179) samples had MG colonies, and 73.2% (131/179) samples had MS colonies. In addition, 11 samples had both MG and MS colonies. Using PCR, a higher number of MG and MS were

detected. *M. gallisepticum* was detected in 138 poultry samples and three non-poultry samples. For MS, 61.2% (301/492) poultry samples and 40.7% (22/54) non-poultry samples were positive by PCR. Twenty-six poultry samples were positive for both MG and MS. Phylogenetic analysis of the MG local isolates showed an identical pattern in both *pvpA* and *mgc2* genes with MG strain F. One of the MG isolates had a different pattern of *mgc2* gene from reference strains. *M. synoviae* field isolates shared an identical pattern of *vlhA* gene with MS strain MS-H. Erythromycin, lincomycin, and chlortetracycline were observed to have the highest number of resistant isolates respectively. The number of positive MG and MS infections detected by either culture or PCR is suggestive of the continuous circulation of these pathogens among birds and poultry of Malaysia. The isolation and characterization of these pathogens in free-flying birds and aviary birds highlighted the possible role of these birds as natural reservoirs. The development of AMR among local isolates of MG and MS can be related to long exposure to antibiotics or unnecessary high antibiotic dosage. Therefore, routine monitoring programs of susceptibility profile of the isolates in order to achieve effective treatment dosage is highly recommended.

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

PEMENCILAN DAN PECIRIAN *Mycoplasma gallisepticum* DAN *Mycoplasma synoviae* PADA POLTRI DAN BURUNG DI SEMENANJUNG MALAYSIA

Oleh

TAIYARI HOSSEIN

Jun 2021

Pengerusi : Profesor Jalila Abu, PhD
Fakulti : Perubatan Veterinar

Mycoplasma gallisepticum (MG) dan *M. synoviae* (MS) terus menyebabkan kerugian ekonomi yang besar kepada industri unggas setiap tahun. Penyakit pernafasan akut dan kronik (CRD), sinusitis, sinovitis, penurunan berat badan yang tinggi, penurunan pengeluaran telur, dan penurunan kadar penetasan adalah sekuel pada mikoplasmosis avian. Kemunculan konjunktivitis mikoplasma pada populasi burung liar menunjukkan kebolehan semula jadi mikoplasma dalam kemampuan menyesuaikan diri dengan pelbagai perumah avian. Keupayaan ini menyumbang kepada reservoir liar baru dan akhirnya penyebaran patogen di persekitaran. Di Malaysia, selain daripada laporan yang menunjukkan prevalen jangkitan MG yang tinggi di ladang ungas komersial dan lepas bebas, satu strain unik MG juga telah dikenal pasti. Di sebalik semua usaha ini, ada kekurangan maklumat mengenai teknik pengesanan optimum (kultur dan PCR), pencirian strain dari pelbagai hos, dan akhirnya, profil kerintangan antibiotik pada isolat lapangan. Oleh itu, kajian ini dilakukan untuk memencilkan, menjalankan pencirian molekul, dan menentukan kerintangan antimikrob isolat MG dan MS dari poltri dan non-poltri di Semenanjung Malaysia. Pengoptimuman kaedah pemencilan dijalankan untuk membandingkan dua jenis media mikoplasma iaitu media Frey bersama serum babi (FMS) dan media PPLO terubahsuai (Chanock). Keputusan menunjukkan peningkatan ketara untuk memencilkan MG dan MS dalam media FMS berbanding media Chanock. Keputusan menunjukkan bahawa MG and MS tumbuh dengan lebih pesat dalam media FMS daripada Chanock. Oleh yang demikian, media FMS digunakan untuk pemencilan. Sebanyak 546 sampel swab celah choanal dikumpulkan dan menjalani pemencilan dan PCR. Menggunakan ujian imunofluoresensi (IFA), 36.3% (198/546) isolat MG and MS dikesan dimana 90.4% (179/198) adalah pencilan daripada poltri, dan 9.6% (19/198) pencilan adalah daripada non-poltri. Bagi sampel non-poltri, 15.8% (3/19) adalah koloni MG, dan 84.2% (16/19) sampel adalah koloni MS. Bagi sampel poltri, 26.8% (48/179) adalah koloni MG, dan 73.2% (131/179) sampel adalah koloni MS. Di samping itu, 11 sampel mempunyai koloni MG dan MS. Menggunakan PCR, bilangan MG dan MS yang lebih tinggi dikesan. *M.gallisepticum* dikesan pada 138 sampel poltri dan tiga sampel non-poltri. Untuk MS, 61.2% (301/492) sampel poltri dan 40.7% (22/54) sampel non-

poltri dikesan positif oleh PCR. Dua puluh enam sampel poltri adalah positif untuk kedua MG dan MS. Analisis filogenetik terhadap isolat tempatan MG menunjukkan profil gen *pvpA* dan *mgc2* yang serupa dengan strain rujukan F. Salah satu isolat MG mempunyai profil gen *mgc2* yang berbeza dari strain rujukan. Isolat lapangan *M. synoviae* mempunyai profil gen *vlhA* yang serupa dengan strain rujukan MS-H. Nilai MIC antimikrob isolat MG dan MS menunjukkan kehadiran kerintangan antimikrob (AMR) di kalangan strain lapangan. Isolat lapangan MG dan MS telah menunjukkan kerintangan terhadap antibiotik mycoplasmasidal yang biasa digunakan, termasuk *tilmicosin, enrofloxacin, erytromicin, lincomycin*, dan *tylosin*. Jumlah MG dan MS positif yang dikesan secara pengkulturan atau PCR menunjukkan penyebaran patogen ini di antara burung dan unggas di Malaysia. Pemencilan dan pencirian patogen ini pada burung lepas bebas dan burung di aviari juga menonjolkan kemungkinan burung ini sebagai takungan semula jadi. Kehadiran AMR di kalangan isolat MG dan MS tempatan boleh dikaitkan dengan program rawatan antibiotik yang kurang teratur. Oleh itu program pemantauan rutin profil kerintangan isolat untuk mencapai dos antimikrob yang efektif untuk rawatan mikoplasmosis burung adalah amat disyorkan.

ACKNOWLEDGEMENTS

Alhamdulillah, all praises and gratitude due to Allah (SWT) for His blessings and strength in completing this thesis.

Firstly, I would like to express my special appreciation and deepest gratitude to my supervisor, Professor Dr. Jalila Abu, for her support, constructive comments, encouragement, and guidance. I will always remember her advice that took me to where I need to go, and her words that made me stronger.

I would like to gratefully thank Professor Dr. Zunita Zakaria, for her valuable guidance, advice, and support. Her suggestions and guidance about isolation, genetics, and antibiotic susceptibility profile works are priceless. I would like to gratefully thank Dr. Nik Mohd Faiz Nik Mohd Azmi, for his support, suggestions, and teaching me about poultry science.

Finally, my deepest gratitude to all my family, especially my dear mother and dear father for their unique spiritual support and encouragement. Words cannot express how grateful I am.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as a fulfilment of the requirement for the degree of Master of Science. The members of the supervisory committee were as follow:

Jalila binti Abu, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Zunita binti Zakaria, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Nik Mohd Faiz bin Nik Mohd Azmi, PhD
Senior Lecturer
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 11 November 2021

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Signature: _____

Name of Chairman of Supervisory Committee: Professor Dr. Jalila binti Abu

Signature: _____

Name of Member of Supervisory Committee: Professor Dr. Zunita binti Zakaria

Signature: _____

Name of Member of Supervisory Committee: Dr. Nik Mohd Faiz bin Nik Mohd Azmi

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LIST OF ABBREVIATIONS

%	Percentage
µg	microgram
µl	microliter
µm	micrometer
°C	Degree in Celsius
AFLP	Amplified Fragment Length Polymorphism
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CO2	Carbon dioxide
CRD	Chronic Respiratory Disease
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide phosphate
DW	Distilled Water
E.coli	*Escherichia coli*
ELISA	Enzyme Linked Immunosorbent Assay
gapA	Adherence protein A
GTS	Gene-Targeted Sequencing
HRM	High-Resolution Melting
IB	Infectious Bronchitis
IgM	Immunoglobin M
kbp	kilo base pair
kDa	kilodalton
L	Liter

MG	*Mycoplasma gallisepticum*
MS	*Mycoplasma synoviae*
mg	milligram
mgc2	Cytadhesion membrane protein
ml	milliliter
mm	millimiter
mM	milli Molar
ND	Newcastle Disease
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCR-RFLP	PCR based Restriction Fragment Length Polymorphism
pvpA	Phase-variable putative adhesin protein
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	radius per minute
rRNA	Ribosomal Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SPA	Serum Plate agglutination
V	Volt
IFA	Immunofluorescence Assay
MIC	Minimum Inhibitory Concentration

CHAPTER 1

INTRODUCTION

1.1 Background

Mycoplasmosis is a bacterial disease caused by bacteria of the genus Mycoplasma. Avian mycoplasmosis is mainly explained as the infections caused by *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma iowae*, and *Mycoplasma meleagridis*. *M. gallisepticum* (MG) can cause acute and chronic respiratory disease (CRD) in birds. Loss of body weight and decrease in egg production and hatchability rate are the results of disease in poultry (Levisohn & Kleven, 2000; Raviv & Ley, 2013). In wild and captive birds, MG has been isolated and mainly causes upper respiratory disease. In 1994, MG caused a conjunctivitis outbreak in house finches, and since then, new species of songbirds have been infected (Ley et al., 1996).

M. synoviae (MS), like MG, mainly causes respiratory system diseases. It could also cause synovitis. Although MG is considered more pathogenic, *M. synoviae* is more contagious; therefore, more prevalent. Apart from poultry species, the co-infection of these two microorganisms was reported in other avian host species such as house finch and scrub jay (Rogers et al., 2019).

The lack of cell wall, extraordinary reduction of the genome, and a minute size distinguish mycoplasma from other bacteria. Mycoplasmas have the smallest size among other prokaryotes (Razin et al., 1998). Adaptation to a variety of hosts and tropism to different tissues play an important role in the metabolism of mycoplasmas and in regulating its austerity. Mucosal surfaces like those available in the respiratory tract are target regions for the colonization of the organism. Remarkable antigenic variation, despite its small genome size, revealed the capability of mycoplasma to survive in immunocompetent hosts (Winner et al., 2000). Limited capacity to synthesize the required nutrients has increased their need for host cells. In this regard, cell surface proteins play a vital role in the adherence of an organism to host cells, leading to the survival of the organism (Bencina, 2002). These surface proteins (lipoproteins) are encoded by cytadhesin genes comprised of *pvpA* and an operon encoding three different genes; *mgc1* (*gapA*), *mgc2*, and *mgc3* (Keeler et al., 1996; Goh et al., 1998; Hnatow et al., 1998; Yoshida et al., 2000). In *M. synoviae*, the *vlhA* gene family is responsible for encoding surface lipoprotein called VlhA (Noormohammadi et al., 1998, 2000). These genes have a great capacity for antigenic variation. The ability to vary the surface components that may function in immune evasion and adaptation to the host environment allows mycoplasma to invade cells and infect new hosts (Bencina, 2002).

1.2 Problem Statement

The first study of avian mycoplasmosis in Malaysia can be traced back to the study carried out by Shah-Majid in 1996, where 26% of the study population were positive for MG using the ELISA test. Recent studies have shown a high prevalence of MG in poultry industries in Malaysia (Yasmin, 2013). The molecular characterization of these poultry isolates showed a unique local strain in Malaysia that differs from other country isolates (Yasmin et al., 2018). The high prevalence of MG (24.2%) and MS (5.7%) were also reported in previous studies conducted in commercial poultry, especially village chickens (Ahmad, 2012). Although new studies could detect the presence of MS using culture and polymerase chain reaction (PCR) techniques, the number of MS positive samples were lower than MG despite MS being considered more prevalent than MG (Olson et al., 1967). Therefore, this study aimed to optimize the detection techniques for MS.

In 2006 and 2007, MG was detected by PCR in free-flying birds , especially those sampled around the poultry farms. This indicated the potential role of free-flying birds in the sustainability of MG (Jaganathan, 2006). These MG infections in free-living birds, along with village chickens, can be caused by either spillover infection as a result of host jump from domesticated poultry or as a natural host for MG as a reservoir or carrier. Unfortunately, none of the studies on free-living birds in Malaysia could isolate the MG, and none of them continued their investigations to the molecular levels (Jaganathan, 2006; Ganapathy et al., 2007).

In addition, despite the application of preventive programs in Malaysia, studies have shown that mycoplasmosis is prevalent among commercial and backyard flocks (Yasmin, 2013). There is no routine monitoring of the antimicrobial susceptibility profile, therefore the antibiotic treatment for mycoplasmosis may not be adequate. The use of live vaccines has been found to pose the risk of virulence recovery of the vaccine strains (Jiang et al., 2009). Phylogenetic analysis and determination of antimicrobial susceptibility profile of the field isolates can distinguish the local strain from vaccine strains that recovered their virulence.

1.3 Research Questions

This study aims to answer the following questions:

1) What is the optimized method for isolation of the Malaysian MG and MS field strains?
2) What are the strain types of MG and MS in Malaysia?
3) What is the genetic relationship between free-flying and poultry MG and MS field isolates?
4) What is the antimicrobial susceptibility profile of Malaysian MG and MS isolates?

1.4 Hypothesis

1) There is a high occurrence of MG and MS infection among commercial chickens.
2) The variation among the MG and MS local strains in different species of birds in Malaysia results from spillover infections, leading to the evolution of more virulent strains.
3) MG and MS are highly resistant to a wide range of antibiotics.

1.5 Research Objectives

1) To compare the growth support of common mycoplasma media and detect MG and MS in poultry and birds using isolation technique and PCR.
2) To perform molecular characterization of field isolates by targeted sequencing of single genomic loci.
3) To determine the antimicrobial susceptibility of MG and MS using microdilution minimum inhibitory concentration (MIC).

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