UNIVERSITI PUTRA MALAYSIA

GENESIZE POLYMORPHISM AND PATHOGENICITY IN EMBRYONATED EGGS OF *MYCOPLASMA GALLISEPTICUM* ISOLATED FROM COMMERCIAL CHICKENS

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BY

TAN CHING GIAP

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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This project paper is especially dedicated to my father, mother, brothers and sisters for their patience, support, encouragements and understanding of my interest in Veterinary Medicine

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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Supervisor: **Professor Aini Ideris, PhD**

Faculty: **Faculty of Veterinary Medicine**

Chronic respiratory disease (CRD) is caused by *Mycoplasma gallisepticum* (MG). Infected birds show respiratory and reproductive problems which lead to severe economic losses in poultry industry. There are only few published data on avian mycoplasmosis in Malaysia, thus, this study was carried out to determine the strain variability and pathogenicity of MG isolates, towards understanding the control of the infection. A total of 4605 samples were collected from chickens and their progeny from various commercial farms using sterile cotton swabs for culturing onto mycoplasma agar. Twenty three (23) MG isolates were obtained from suspected MG infected commercial chickens. Although MG could be isolated from various sites of the host, in this study, choanal and tracheal sites proved to be the best sites in live birds. On the other hand, trachea and airsac samples were the best sites for the detection of MG for chick embryos or chicks. Size variations among polymerase chain reaction products divergence of the MG-specific gene were the basis for strain differentiation. The local isolates exhibited gene size polymorphism in *pvp*A gene, 16S–23S rRNA intergenic spacer region gene, *Crm*A gene and *pMGA* or *vlh*A gene

with the presence of insertion or deletion observed in PCR products. However, the *gap*A gene, LP gene, F-strain-specific DNA fragment gene, *Crm*B gene, *Crm*C gene, p47 gene, HMW3-like protein gene and *pneumoniae*-like protein A gene sequences were constant in size. The embryonated eggs were each inoculated with "pleuropneumonia like organism" (PPLO) broth containing MG strains, via yolk sac. *Mycoplasma gallisepticum* embryos, broth inoculated and uninoculated control embryonated eggs were examined at necropsy days 7, 10, 13 and 15 post-inoculation. The pathogenicity of the isolates in chicken embryonated eggs showed variations among each other. The MG isolates and strains that showed a wide variation in genes were examined for virulence in ovo. In this study, the presence of caseous airsac lesion correlated with virulence of MG and presence of high maternal antibody titer. MG were isolated only in embryos that did not develop any caseous airsac lesions. MG inoculated embryos were polymerase chain reaction (PCR) positive regardless of the absence or presence of caseous airsac lesion, suggesting that caseous airsac lesion maybe the result of formation of antigen-antibody complex. Caseous airsacs were found to be one of the prominent lesions associated with MG infection. For certain highly pathogenic strains, there was clear relationship between the caseous airsac lesion and the presence of maternal antibody titer and embryo mortality. Less pathogenic strains that grow well usually caused embryo mortality during later stages of incubation and there was no strict correlation between caseous airsac lesion and the presence or absence of maternal antibody and embryo mortality. Based on the presence of the gene size polymorphism in *pvp*A gene and *pMGA* or *vlh*A gene; MGS6 (reference strain), I44 and I-18 strains of MG showed a similar pattern of pathogenicity in that they are highly pathogenic, whereas, H21 8T, H21 11T, H24 5C and H26 9C have similar pattern of molecular characterization and pathogenicity

with ts-11 (vaccine strain), characterized by their less pathogenicity in embryos. MGS6, I44 and I-18 strains caused early embryonic death compared to ts-11, H21 8T, H21 11T, H24 5C and H26 9C strains that caused embryo mortality during later stages of incubation. At this point, the postulation is that, when maternal antibody of MG is high and MG challenge is present, caseous airsac may occur. This would be due to maternal antibody in the eggs which may bind to MG that served as antigen to form antigen-antibody complexes. The immune complexes may help to release cytokines and attract more macrophages and other inflammatory cells, which help to increase the severity of the air sac lesion. When the MG strain with the gene size polymorphism in *pvp*A gene and *pMGA* or *vlh*A gene that has similar pattern with MGS6, it correlates with the formation of caseous airsac, as well as the increase in severity of the caseous airsac. This study showed that the combination of the gene size polymorphism in *pvp*A gene and *pMGA* or *vlh*A gene can be used as pathogenic markers for MG in determination of its pathogenicity towards chick embryos. Based on characterization and pathogenicity, MG field strains H21 8T, H21 11T, H24 5C and H26 9C showed similar pattern of molecular and pathogenicity characteristic with ts-11 and therefore are potential candidates for live MG vaccine.

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

POLYMORPHISM SAIZ GEN DAN PATOGENISITI *MYCOPLASMA GALLISEPTICUM* DALAM TELUR AYAM BEREMBRIO DARIPADA LADANG AYAM KOMERSIAL

Oleh

TAN CHING GIAP

August 2008

Pengerusi: **Profesor Aini Ideris, PhD**

Fakulti: **Fakulti Perubatan Veterinar**

Penyakit pernafasan kronik (CRD) adalah disebabkan oleh *Mycoplasma gallisepticum* (MG). Ayam yang telah dijangkiti menunjukkan tanda-tanda masalah pernafasan dan reproduktif yang memberi kerugian yang besar pada industri ayam. Hanya sedikit data yang telah diterbitkan berkenaan dengan mycoplasmosis unggas di Malaysia. Oleh itu kajian ini dilakukan untuk mengenalpasti kebarangkalian variasi dan patogenik isolat-isolat MG ke arah memahami cara-cara mengawal jangkitan. Sebanyak 4605 sampel swab daripada ladang-ladang komersial dikultur ke dalam agar. Dua puluh tiga isolat MG telah berjaya diperoleh daripada ladang ayam komersial yang disyaki telah dijangkiti MG. Dalam kajian ini, choanal dan trakea merupakan bahagian yang terbaik untuk mendapatkan isolat MG dari unggas hidup, walaupun MG boleh didapati di pelbagai bahagian perumah. Bagi mengesan MG pada embrio ayam dan anak ayam, bahagian terbaik pula merupakan trakea dan katung pernafasan. Perbezaan saiz antara bahan hasil proses 'polymerase chain reaction' di antara gen tertentu dalam MG adalah asas untuk pembezaan strain. Isolat tempatan mempamerkan polymorphism saiz gen bagi gen *pvp*A, gen 16S–23S rRNA

intergenic spacer region, gen *Crm*A dan gen *pMGA* atau *vlh*A dengan kehadiran insertion ataupun deletion yang dapat diperhatikan di dalam produk PCR. Walaubagaimanapun, gen *gap*A, gen LP, gen F-strain-specifik DNA fragment, gen *Crm*B, gen *Crm*C, gen p47, gen HMW3-like protein dan gen *pneumoniae*-like protein A menunjukkan saiz yang konsistan. Setiap telur berembrio disuntik dengan "pleuropneumonia like organism" (PPLO) yang mengandungi MG strain melalui kantung kuning telur. Telur berembrio yang disuntik dengan *Mycoplasma gallisepticum*, telur dari kumpulan broth dan telur berembrio yang tidak disuntik, diperiksa semasa nekropsi pada hari 7, 10,13 dan 15 selepas hari suntikan. Patogenisiti isolat-isolat yang terdapat pada embrio ayam mempamerkan variasi antara satu sama lain. Isolat-isolat dan strain MG yang menunjukkan variasi diperiksa patogenisitinya in ovo. Dalam kajian ini, kehadiran lesi kaseous katung pernafasan dikaitkan dengan patogenisiti MG dan kehadiran titer maternal antibodi yang tinggi. MG hidup hanya dapat dikultur dari embrio yang telah diinokulasi dengan MG dan tidak membentuk ataupun menghasilkan lesi kaseous katung pernafasan yang berskala satu. Embrio yang telah diiokulasi sama ada dengan atau tiada penghasilan kaseous katung pernafasan memberikan keputusan PCR yang positif, menyarankan bahawa lesi kaseous katung pernafasan berkemungkinan terhasil daripada pembentukan komplek antigen-antibodi. Kaseous katung udara didapati merupakan lesi yang paling ketara dikaitkan dengan jangkitan MG. Bagi sebilangan strain yang amat patogenik, terdapat hubungkait yang jelas di antara lesi kaseous katung pernafasan dan kehadiran titer matermal antibodi serta kematian embrio. Strain-strain kurang patogenik yang tumbuh dengan baik biasanya menyebabkan kematian embrio pada peringkat akhir inkubasi dan tiada hubungkait yang jelas di antara lesi kaseous katung pernafasan dan kehadiran maternal antibodi

dan kematian embryio. Berpandukan kepada kehadiran polymorphism saiz gen pada gen *pvp*A dan gen *pMGA* atau *vlh*A, MGS6 (strain rujukan), I44 dan I-18 menunjukkan corak patogenisiti yang sama di mana kesemuanya adalah amat patogenik. Manakala ts-11 (strain vaksin), H21 8T, H21 11T, H24 5C dan H26 9C memiliki corak patogenisiti yang sama dikategorikan oleh patogenisiti yang lebih rendah pada embrio. Strain-strain MGS6, I44 dan I-18 menyebabkan kematian embrio pada awal inkubasi berbanding dengan strain-strain ts-11, H21 8T, H21 11T, H24 5C dan H26 9C pada peringkat-peringkat akhir inkubasi. Pada tahap ini, andaian yang dibuat ialah apabila maternal antibodi bagi MG tinggi dan dengan kehadiran MG, kaseous katung pernafasan mungkin berlaku. Ini disebabkan oleh maternal antibodi dalam telur yang mana boleh terikat pada MG yang bertindak sebagai antigen untuk membentuk antigen-antibodi kompleks. Kompleks imun boleh membantu untuk membebaskan sitokin dan menarik lebih makrofaj dan sel radang yang lain, yang mana membantu untuk meningkatkan tahap keterukan lesi katung pernafasan. Apabila strain MG yang memiliki polymorphism saiz gen pada gen-gen *pvp*A dan *pMGA* atau *vlh*A yang meyerupai dengan MGS6, ini akan berhubungkait dengan pembentukan kaseous katung pernafasan dan juga terlibat dengan peningkatan tahap severiti kaseous katung pernafasan. Kajian ini menunjukkan bahawa kombinasi polymorphism saiz gen-gen *pvp*A dan *pMGA* atau *vlh*A boleh digunakan sebagai penanda patogenik MG dalam penentuan patogenisitinya terhadap embrio ayam. Berdasarkan ciri-ciri gen dan patogenisiti, strain tempatan MG H21 8T, H21 11T, H24 5C and H26 9C telah menunjukkan corak molekular dan ciri-ciri patogenisiti yang sama dengan ts-11 dan merupakan calon-calon yang berpotensi sebagai vaksin hidup MG.

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I certify that the Examination Committee met on 25 August 2008 to conduct the final examination of Tan Ching Giap on his Master of Veterinary Science thesis entitled "Gene Size Polymorphism and Pathogenicity in Embryonated Eggs of *Mycoplasma gallisepticum* Isolated from Commercial Chicken" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Veterinary Science.

Members of the Examination Committee were as follows:

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Date: 23 October 2008

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

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Date: 13 November 2008

DECLARATION

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 concurrently submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

Tan Ching Giap

Date: 26 September 2008

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List of Abbreviations

%	Percentage
AFLP	Amplified fragment length polymorphism
AP-PCR	Arbitrary primed PCR
BBC	Broiler breeder chicken
BC	Broiler chicken
bp	Base pair
CAM	Chorio allantoic membrane
CCRD	Complicated chronic respiratory disease
CCU	Color changing unit
CFU	Colony forming unit
cm	Centimeter
CO_2	Carbon dioxide
CRD	Chronic respiratory disease
CrmA	Cytadherence-related molecule A
CrmB	Cytadherence-related molecule B
CrmC	Cytadherence-related molecule C
DDW	Double distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein isothiocyanate
gapA	Adherence protein A
GTS	Gene-targeted sequencing
HI	Hemagglutination inhibition
I	Infected
IBV	Infectious bronchitis virus
IFA	Immunofluorescence antibody
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGSR	16S-23S rRNA intergenic spacer region sequencing

kbp	kilobase pairs
kD	kilo Daltons
LP	Surface lipoprotein
MG	*Mycoplasma gallisepticum*
mg	milligram
mgc2	Cytadhesin membrane protein
$MgCl_2$	Magnesium chloride
ml	milliliter
mm	millimeter
mM	milli Molar
MS	*Mycoplasma synoviae*
N	Normal
NC	Normal chick
NDV	Newcastle disease virus
ng	Nanogram
nm	nanometer
°C	Degree in Celsius
p47	Macrophage-activating lipoprotein-like protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	PCR based restriction fragment length polymorphism
PE	Pipped embryo
PFGE	Pulsed field gel electrophoresis
pH	Logarithm 10 {H}
PI	Post inoculation
pMGA	Hemagglutinin protein
pmole	Picomole
PPLO	Pleuropneumonia like organism
pvpA	Phase-variable putative adhesin protein
RAPD	Random amplified polymorphic DNA
REA	Restriction endonuclease analysis
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid