UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A DIAGNOSTIC REAL TIME PCR ASSAY FOR MOLECULAR DETECTION AND CHARACTERIZATION OF MYCOPLASMA GALLISEPTICUM

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By

FARHANA YASMIN

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

September 2013

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DEDICATION

This thesis is dedicated to my beloved grandparents

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

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September 2013

Chairman: **Professor Datin Paduka Aini Ideris, PhD**

Faculty: **Veterinary Medicine**

Mycoplasma gallisepticum (MG) causes chronic respiratory disease leading to huge economic losses to poultry industry worldwide. Early and efficient detection is therefore crucial in reducing the loss sustained by poultry farmers and poultry industry at large. This study was carried out to develop SYBR green Real Time Polymerase Chain Reaction (PCR) for the detection of MG and to perform molecular characterization of local MG strains based on gene targeted sequencing (GTS) analysis. A Real Time PCR assay was developed using primer specific to *gapA* gene. The primer was able to amplify the expected DNA fragment of 505 bp. The assay was found to be specific and highly sensitive in detecting MG as indicated by its ability to detect MG at early cycle of amplification with 26.05 Cq value in comparison with other avian Mycoplasma species as well as the ability of detecting 260ng/µl to 26pg/µl of DNA template. A total of 300 swab samples were collected from different poultry farms (layer breeder, broiler breeder, broiler chicken and

village chicken) in Peninsular Malaysia and tested for the presence of MG using the developed PCR assay. Of the total number of samples tested, 31% (94/300) were found to be positive for MG, thus, indicating high prevalence of MG infection in many distinct geographical areas of the country. Although, farmers vaccinate and treat the chickens against MG in Malaysia, the results of the present study suggest that the control of MG might not be efficient. In order to gain further observation on the molecular characteristics of Malaysian MG strains detected in this study, selected gene target specific sequences to MG, hemagglutinin protein A gene (*pMGA*) and phase variable putative adhesion protein A gene (*pvpA*), were amplified from positive MG samples using conventional polymerase chain reaction. A total of 25 MG positive field samples out of 94 MG samples were sequenced with the primer targeting the *pMGA* and also *pvpA* gene. The sequencing and phylogenetic analysis were conducted using bioinformatics software (Bioedit and MEGA 5 Software) and genetic variation patterns were evaluated based on partial nucleotide sequencing of the *pMGA* and *pvpA* genes. In case of *pMGA* partial nucleotide sequences, all 25 local field strains showed similar gene size pattern with pathogenic reference strain, MGS6 and pathogenic vaccine strain, MG F which is different from the PCR product size of less pathogenic vaccine strain, TS 11. In the same way, *pvpA* partial nucleotide sequences of MG local strains showed that 20 out of 25 local strains possess similar gene size pattern with MG F, which is different from MGS6 and TS 11. From the phylogenetic analysis of *pMGA* and *pvpA* partial nucleotide sequences, it was found that local (Malaysia) MG strains are different from the strains reported in other countries (Australia, USA, Iran, Israel, China and Russia).

In conclusion, this study successfully developed a specific and sensitive real time PCR assay for the rapid detection of MG compared to conventional PCR method. Although the cost to carry out real time PCR is more expensive, it is more specific, sensitive, and more rapid for detection of MG as compared with conventional PCR. With more research, the time and cost factors can be improved. This method gave result within an hour but conventional PCR took 3 hrs excluding post PCR processing. Based on the *pMGA* and *pvpA* partial nucleotide sequence analysis and phylogenetic tree it was shown that local MG strains were different from strains reported in other countries (Australia, USA, Iran, Israel, China and Russia).

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

PEMBANGUNAN ASAI "REAL TIME PCR" DIAGNOSTIK UNTUK PENGESANAN MOLEKULAR DAN PENCIRIAN *MYCOPLASMA GALLISEPTICUM*

Oleh

FARHANA YASMIN

September 2013

Pengerusi: Professor Datin Paduka Aini Ideris, PhD

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Mycoplasma gallisepticum (MG) menyebabkan penyakit respirasi kronik yang membawa kepada kerugian ekonomi yang besar kepada industri ternakan ayam di seluruh dunia. Pengesanan awal dan berkesan adalah sangat penting dalam mengurangkan kerugian yang dialami oleh penternak ayam dan industri ternakan ayam secara amnya. Kajian ini dijalankan bagi membangunkan "Real Time Polymerase Chain Reaction" (PCR) bagi pengesanan MG dan menjalankan pencirian molekular isolat-isolat MG tempatan. Suatu asai "Real Time PCR" telah dibangunkan menggunakan primer khusus terhadap gen *gapA*. Primer ini mampu mengamplifikasi fragmen DNA yang dijangkakan sebesar 505bp. Asai tersebut didapati adalah spesifik dan amat sensitif di dalam pengesanan MG sebagaimana yang ditunjukkan dalam keupayaannya untuk mengesan antara 260ng/µl hingga 26pg/µl templat DNA. Sejumlah 300 sampel swab telah dikumpulkan dari ladang-ladang ternakan ayam yang berbeza (pembiak baka ayam penelur,

pembiak baka ayam pedaging, ayam pedaging dan ayam kampong) di Semenanjung Malaysia dan diuji untuk kehadiran MG menggunakan asai PCR yang telah dibangunkan. Daripada sejumlah sampel yang diuji, 31% (95/300) didapati positif bagi MG, di mana ia menunjukkan penyebaran jangkitan MG yang luas di kebanyakan kawasan yang mempunyai kedudukan geografi yang berbeza di negara ini. Walaupun penternak menyuntik dan merawat ayam-ayam terhadap MG di Malaysia, keputusan kajian ini menggambarkan bahawa kawalan terhadap MG mungkin tidak berkesan.

Bagi mendapatkan pemerhatian terperinci tentang ciri-ciri molekular isolat-isolat MG Malaysia yang dikesan dalam kajian ini, gen sasaran dengan urutan spesifik terhadap MG terpilih, gen hemagglutinin protein A (pMGA) dan gen fasa pelekatan boleh-ubah putatif protein A (pvpA) telah diamplifikasi daripada sampel-sampel MG positif menggunakan tindak balas berantai polimerase konvensional. Sejumlah 25 sampel lapangan positif MG daripada 94 sampel-sampel MG telah dijujukkan menggunakan primer yang mensasarkan gen pMGA dan juga gen pvpA. Penjujukan dan analisa filogenetik telah dijalankan menggunakan perisian bioinformatik (Bioedit and MEGA 5 Software) dan pola variasi genetik dinilai berdasarkan penjujukan separa nukleotida gen-gen pMGA dan pvpA. Dalam kajian penjujukan separa nukleotida pMGA, kesemua 25 isolat lapangan tempatan menunjukkan pola saiz gen yang sama dengan strain patogenik rujukan, MGS6 dan strain vaksin patogenik, MG F yang mana berbeza daripada saiz produk PCR strain vaksin yang kurang patogenik, TS 11. Dengan cara yang serupa, penjujukan separa nukleotida pvpA isolat tempatan menunjukkan 20 daripada 25 isolat-

isolat tempatan mempunyai pola saiz gen yang sama dengan MG F, yang berbeza dari MGS6 dan TS 11. Daripada analisa filogenetik jujukan separa nukleotida pMGA dan pvpA, didapati bahawa isolat MG tempatan (Malaysia) adalah berbeza daripada isolat-isolat yang dilaporkan di negara-negara lain (Australia, USA, Iran, Israel, China dan Russia).

Sebagai kesimpulan, kajian ini berjaya membangunkan suatu asai “Real Time PCR” yang spesifik dan berkesan bagi pengesanan segera MG berbanding kaedah PCR konvensional kerana kaedah ini. Walaupun kos untuk menjalankan “real time PCR” lebih mahal, ianya adalah lebih spesifik, sensitive, dan berupaya mengesan MG dengan lebih cepat berbanding PCR konvensional. Dengan kajian lanjut, factor-factor masa dan kos boleh diperbaiki. Kaedah ini memberikan keputusan dalam masa satu jam tetapi PCR konvensional mengambil masa tiga jam tidak termasuk pemprosesan selepas PCR. Berdasarkan penjujukan separa nukleotida dan analisa filogenetik gen-gen *pMGA* dan *pvpA* menun jukkan isolate MG tempatan adalah berbeza daripada isolate-isolat yang telah dilaporkan di negara – negara lain (Australia, USA, Iran, Israel, China dan Russia).

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I certify that a Thesis Examination Committee has met on 27 September 2013 to conduct the final examination of Farhana Yasmin on her thesis entitled " Development of a Diagnostic Real Time PCR Assay for Molecular Detection and Characterization of *Mycoplasma gallisepticum*" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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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 Science. The members of the supervisory committee were as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations that have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

FARHANA YASMIN

Date: 27 September 2013

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