



UNIVERSITI PUTRA MALAYSIA

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

TAN CHING GIAP

FPV 2008 14

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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

August 2008



**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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EMBRYONATED EGGS OF *MYCOPLASMA GALLISEPTICUM* ISOLATED
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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 *pvpA* gene, 16S–23S rRNA intergenic spacer region gene, *CrmA* gene and *pMGA* or *vlhA* gene



with the presence of insertion or deletion observed in PCR products. However, the *gapA* gene, LP gene, F-strain-specific DNA fragment gene, *CrmB* gene, *CrmC* 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 *pvpA* gene and *pMGA* or *vlhA* 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 *pvpA* gene and *pMGA* or *vlhA* 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 *pvpA* gene and *pMGA* or *vlhA* 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

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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 diperolehi 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 *pvpA*, gen 16S-23S rRNA



intergenic spacer region, gen *CrmA* dan gen *pMGA* atau *vlhA* dengan kehadiran insertion ataupun deletion yang dapat diperhatikan di dalam produk PCR. Walaubagaimanapun, gen *gapA*, gen LP, gen F-strain-specific DNA fragment, gen *CrmbB*, gen *CrmbC*, gen p47, gen HMW3-like protein dan gen *pneumoniae*-like protein A menunjukkan saiz yang konsisten. 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 diinokulasi sama ada dengan atau tiada penghasilan kaseous katung pernafasan memberikan keputusan PCR yang positif, menyarankan bahawa lesi kaseous katung pernafasan berkemungkinan terhasil daripada pembentukan kompleks 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 maternal 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 embrio. Berpandukan kepada kehadiran polymorphism saiz gen pada gen *pvpA* dan gen *pMGA* atau *vlhA*, MGS6 (strain rujukan), I44 dan I-18 menunjukkan corak patogenesisiti yang sama di mana kesemuanya adalah amat patogenik. Manakala ts-11 (strain vaksin), H21 8T, H21 11T, H24 5C dan H26 9C memiliki corak patogenesisiti yang sama dikategorikan oleh patogenesisiti 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 *pvpA* dan *pMGA* atau *vlhA* 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 *pvpA* dan *pMGA* atau *vlhA* boleh digunakan sebagai penanda patogenik MG dalam penentuan patogenesisitinya terhadap embrio ayam. Berdasarkan ciri-ciri gen dan patogenesisiti, strain tempatan MG H21 8T, H21 11T, H24 5C and H26 9C telah menunjukkan corak molekular dan ciri-ciri patogenesisiti yang sama dengan ts-11 dan merupakan calon-calon yang berpotensi sebagai vaksin hidup MG.

ACKNOWLEDGEMENTS

I would like to express my heartiest gratitude and appreciation to my supervisor, Prof. Aini Ideris, for her advice, encouragement, time and guidance throughout this project.

I would like to express my thanks and appreciation to my co-supervisors, Assoc. Prof. Dr. Abdul Rahman and Prof Dr. Mohd Hair Bejo for their precious suggestions and assistance during this study. My thanks also go to Dr. Jalila Abu for her precious suggestions and assistance during this study.

I would like to thank Mdm Tan Lin Ji from Veterinary Research Institute (VRI), Ipoh, for her advice, encouragement, time and guidance throughout this project. The study would not have been possible without her generosity and cooperation.

My appreciation also extends to Regents' Professor Emeritus Dr. S.H. Kleven from Poultry Diagnostic and Research Center (PDRC) of University of Georgia, Athens and the research team members, Ms. Victoria Leiting and Ms. Ruth Wooten for their willingness to share their vast knowledge and experience in *Mycoplasma gallisepticum*. I am also indebted to Professor Dr. Pedro Villegas from Poultry Diagnostic and Research Center (PDRC) of University of Georgia, Athens, Dr. Somsak Pakpinyo from Faculty of Veterinary Science, Chulalongkorn University, Thailand, Dr. Severine Tasker from University of Bristol, UK, Dr. Wisanu Wanasawaeng from Sahafarm Co. Ltd., Thailand, Dr. Chavalit Piriyaabenjawat from ELANCO, Thailand, Dr. Chris Morrow from Bioproperties Ltd, Australia, Dr. Raymond Choo from Rhone Ma Sdn. Bhd, Malaysia, and Dr. Kok Poe Chu from Sunzen Corporation Sdn. Bhd., Malaysia, in which, this study would be less meaningful without their support.



My thanks also go to my seniors especially Dr. Mah Choew Kong, Dr. Tan Joo Tiam, Dr. Ng Hon Yean, Dr. Tang Siew Ching, Dr. Goh Yong Meng, Dr. Hoo Chun Howe, Dr. Lai Fui Chong, Dr. Anna Wong, Dr. Lim Woe Meng, Dr. Phang Yuen Fun, Dr. Lee Lai Hsiang, Dr Koh Thong Jin, Dr. Ong Eet Ling and to everyone who have helped me during this study.

My appreciation is also extended to the entire postgraduate students in Biologics Laboratory, Faculty of Veterinary Medicine, UPM and Ms. Siti Khatijah in sharing their technical knowledge and advices, as well as their patience and tolerance towards my completion of this study.

I also would like to thank all the farm owners who allowed me to take samples from their farms, helped, guided and made the sampling possible. Not forgetting, the farm supervisors and workers who patiently helped to catch chickens during sampling. Last but not least, I would like to thank all individuals who were directly or indirectly involved in this project. Thank you also to my friends who always gave me suggestions and ideas regarding my project and spent time with me whenever I faced difficulties in the project.



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.

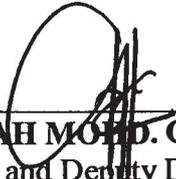
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DECLARATION

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

TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL	xi
DECLARATION	xiii
LIST OF TABLES	xvi
LIST OF FIGURES	xvii
LIST OF PLATES	xxi
LIST OF ABBREVIATIONS	xxiii
CHAPTERS	
I INTRODUCTION	1
II LITERATURE REVIEW	
Mycoplasma	6
Avian Mycoplasmosis	8
Chronic Respiratory Disease (<i>Mycoplasma gallisepticum</i>)	9
Strain Classification	10
Antigenicity of <i>Mycoplasma gallisepticum</i>	10
Genetic or Molecular Characteristics of <i>Mycoplasma gallisepticum</i>	12
Epidemiology	14
Host	14
Transmission	15
Influencing Factor for <i>Mycoplasma gallisepticum</i> Outbreak	15
Economic Impact	16
Clinical signs	17
Pathogenesis	19
Gross Lesions	20
Microscopic Lesions	21
Diagnosis	22
Isolation and Identification of Causative Agent	22
Serology	24
Intervention Strategies: Control and Prevention	26
Management Procedures	26
Immunization	28
III DETECTION AND MOLECULAR CHARACTERISATION OF MYCOPLASMA GALLISEPTICUM	
Introduction	31
Materials and Methods	35
Samplings	35
Commercial Chicken Farms	35
Pipped embryos, poor quality chicks and normal chicks	36
Detection of <i>Mycoplasma gallisepticum</i>	40
Culture	40
Identification of <i>Mycoplasma gallisepticum</i>	41
Immunofluorescence Antibody Test	41
Polymerase Chain Reaction	42
DNA Isolation	42



	Quantification of the Concentration and Purity of the DNA	43
	Conventional Polymerase Chain Reaction	44
	Molecular Characterization of <i>Mycoplasma gallisepticum</i>	45
	<i>Mycoplasma gallisepticum</i> Strains	45
	DNA Isolation	46
	Primers Selection	46
	Polymerase Chain Reaction Targeted Gene	46
	pvpA, gapA, mgc2, LP, pMGA or vfhA genes family, CrmA, CrmB, CrmC, p47, HMW3-like protein, pneumoniae-like protein A, F-strain-specific DNA fragment, M9 and 16S-23S rRNA Intergenic Spacer Region	
	Results	50
	Discussion	83
	Conclusion	91
IV	PATHOGENICITY OF <i>MYCOPLASMA GALLISEPTICUM</i> IN EMBRYONATED EGGS	
	Introduction	92
	Materials and Methods	94
	<i>Mycoplasma gallisepticum</i> strains	94
	Determination of Inoculum	95
	Embryonated Chicken Eggs	96
	Specific Pathogen Free Eggs	96
	Commercial Farms Eggs	96
	Different Sources Eggs	96
	Egg inoculation	97
	Sampling	98
	Gross Lesions Examination	98
	Microscopic Examination	99
	Verification of <i>Mycoplasma gallisepticum</i> strain	99
	Polymerase Chain Reaction	101
	Antibody extraction from egg yolk	101
	Enzyme linked immunosorbent assay	102
	Statistical Analysis	103
	Results	104
	Discussion	154
	Conclusion	158
V	GENERAL DISCUSSION AND CONCLUSION	160
	BIBLIOGRAPHY	166
	APPENDICES	191
	BIODATA OF STUDENT	241
	LIST OF PUBLICATIONS	243



LIST OF TABLES

Table		Page
2.2	Characteristics of avian mycoplasmas	8
3.2.1	Farms samples	35
3.2.2a	Pipped embryos samples	37
3.2.2b	Poor quality chicks samples	38
3.2.2c	Normal chicks samples	38
3.3.3.4a	The nucleotide sequences of universal primers	44
3.3.3.4b	Reagents used in conventional PCR master mixture reaction	45
3.4.3a	Product sizes and sequence positions for primers used in GTS analysis	47
3.4.3b	Reagents used in charaterisation primer for PCR master mixture reaction	49
3.5.1	Caseous airsacs	51
3.5.4a	The presence or absence of gene size polymorphism by using various primer sets	78
3.5.4b	The detail of presence or absence of gene size polymorphism by using various primer sets	80
4.2.1	Mycoplasma strains	95
4.2.8	Reagents used in PCR master mixture reaction.	101
4.3.2.1a	Caseous air sac in MG inoculated SPF embryos or day old chicks	114
4.3.2.1b	Caseous air sac in MG inoculated commercial embryos at day 15 pi	115
4.3.2.1c	Caseous air sac in MG inoculated embryos from different sources at day 15 pi	117
4.3.2.2a	Green liver of SPF embryo with MG inoculation at day 7, 10 and 13 pi	118
4.3.2.2b	Green liver of commercial embryo with MG inoculation at day 7 and 10 pi	119
4.3.2.2c	Green liver of different sources embryo with MG inoculation at day 7 and 10 post inoculation	120
4.3.3a	Percentage of hatchability in SPF embryos	124
4.3.3b	Percentage of hatchability in commercial chicken embryos	125
4.3.3c	Percentage of hatchability of chicken embryos in different sources	126
4.3.4	ELISA test result of different sources of eggs	127



LISTS OF FIGURES

Figure		Page
3.5.3	PCR product of 180bp of MG isolates amplified using the universal primer set (16S ribosomal)	55
3.5.4.1.	PCR product of 980bp of MG isolates amplified using the MGC/GAPA 1F + 1R primer set	56
3.5.4.2	PCR product of 332bp of MG isolates amplified using the Myc 3F + 4R primer set	57
3.5.4.3	PCR product of 505bp of MG isolates amplified using the gapA 5F + 6R primer set	57
3.5.4.4	PCR product of 732bp of MG isolates amplified using the Mgc 1F + 2R primer set	58
3.5.4.5	PCR product of 590bp of MG isolates amplified using the lp 1F + 1R primer set	59
3.5.4.6	PCR product of 349bp of MG isolates amplified using the LP 3F + 4R primer set	59
3.5.4.7	PCR product of ~702bp of MG isolates amplified using the pvpA 1F + 2R primer set	60
3.5.4.8	PCR product of ~402bp of MG isolates amplified using the pvpA 3F + 2R primer set	61
3.5.4.9	PCR product of 824bp of MG isolates amplified using the mgc2 1F + 1R primer set	62
3.5.4.10	PCR product of 770bp of MG isolates amplified using the Mgc2 F1 + R1 primer set	62
3.5.4.11	PCR product of 524bp of MG isolates amplified using the MGF-PIL + PIR primer set	63
3.5.4.12	PCR product of 774bp of MG isolates amplified using the CrmA-F6 + R8 primer set	64
3.5.4.13	PCR product of ~602bp of MG isolates amplified using the CrmA-F5 + R7 primer set	65
3.5.4.14	PCR product of ~555bp of MG isolates amplified using the CrmA-F7 + R9 primer set	65
3.5.4.15	PCR product of 736bp of MG isolates amplified using the CrmB-F3 + R8 primer set	66
3.5.4.16	PCR product of 647bp of MG isolates amplified using the CrmC-F1 + R1 primer set	67
3.5.4.17	PCR product of 812bp of MG isolates amplified using the MG IGSR F + R primer set	68
3.5.4.18	PCR product of ~1057bp of MG isolates amplified using the 16 F +	69



	R primer set	
3.5.4.19	PCR product of 219bp of MG isolates amplified using the MG1273f + 1427r primer set	69
3.5.4.20	PCR product of 1935bp of MG isolates amplified using the pMGA Fo + Ro primer set	70
3.5.4.21	PCR product of 500bp of MG isolates amplified using the pMGA F1i + R1i primer set	71
3.5.4.22	PCR product of ~329bp of MG isolates amplified using the AU TS11 F + R primer set	71
3.5.4.23	PCR product of ~336bp of MG isolates amplified using the M9 F + R primer set	72
3.5.4.24	PCR product of 1200bp of MG isolates amplified using the p47 BQF + BSR primer set	73
3.5.4.25	PCR product of 400bp of MG isolates amplified using the p47 BRF + BGR primer set	74
3.5.4.26	PCR product of 1000bp of MG isolates amplified using the hlp3 F SG1083 + R SG1084 primer set	75
3.6.4.27	PCR product of 2450bp of MG isolates amplified using the <i>plpA</i> F SG1182 + R SG1183 primer set	76
4.3.1a	Mean weight of SPF embryos inoculated with various strains of MG sampled at various time	105
4.3.1b	Mean weight of commercial embryos inoculated with various strains of MG sampled at various time	106
4.3.1c.1	Mean weight of SPF embryos (different sources) inoculated with various strains of MG sampled at various time	107
4.3.1c.2	Mean weight of Farm A embryos inoculated with various strains of MG sampled at various time	109
4.3.1c.3	Mean weight of Farm B embryos inoculated with various strains of MG sampled at various time	110
4.3.1c.4	Mean weight of Farm C embryos inoculated with various strains of MG sampled at various time	112
4.3.5a.1	Histological lesion scoring of SPF embryonated chicken eggs inoculated with various strains of MG sampled at day 7 pi	129
4.3.5a.2	Histological lesion scoring of SPF embryonated chicken eggs inoculated with various strains of MG sampled at day 10 pi	130
4.3.5a.3	Histological lesion scoring of SPF embryonated chicken eggs inoculated with various strains of MG sampled at day 13 pi	132
4.3.5a.4	Histological lesion scoring of SPF embryonated chicken eggs inoculated with various strains of MG sampled at day 15 pi	133

4.3.5b.1	Histological lesion scoring of commercial embryonated chicken eggs inoculated with various strains of MG sampled at day 7 pi	135
4.3.5b.2	Histological lesion scoring of commercial embryonated chicken eggs inoculated with various strains of MG sampled at day 10 pi	136
4.3.5b.3	Histological lesion scoring of commercial embryonated chicken eggs inoculated with various strains of MG sampled at day 13 pi	137
4.3.5b.4	Histological lesion scoring of commercial embryonated chicken eggs inoculated with various strains of MG sampled at day 15 pi	138
4.3.5c.1	Histological lesion scoring of SPF embryonated chicken eggs (different sources) inoculated with various strains of MG sampled at day 7 pi	141
4.3.5c.2	Histological lesion scoring of SPF embryonated chicken eggs (different sources) inoculated with various strains of MG sampled at day 10 pi	141
4.3.5c.3	Histological lesion scoring of SPF embryonated chicken eggs (different sources) inoculated with various strains of MG sampled at day 13 pi	142
4.3.5c.4	Histological lesion scoring of SPF embryonated chicken eggs (different sources) inoculated with various strains of MG sampled at day 15 pi	142
4.3.5c.5	Histological lesion scoring of embryonated chicken eggs from Farm A (different sources) inoculated with various strains of MG sampled at day 7 pi	144
4.3.5c.6	Histological lesion scoring of embryonated chicken eggs from Farm A (different sources) inoculated with various strains of MG sampled at day 10 pi	144
4.3.5c.7	Histological lesion scoring of embryonated chicken eggs from Farm A (different sources) inoculated with various strains of MG sampled at day 13 pi	145
4.3.5c.8	Histological lesion scoring of embryonated chicken eggs from Farm A (different sources) inoculated with various strains of MG sampled at day 15 pi	145
4.3.5c.9	Histological lesion scoring of embryonated chicken eggs from Farm B (different sources) inoculated with various strains of MG sampled at day 7 pi	147
4.3.5c.10	Histological lesion scoring of embryonated chicken eggs from Farm B (different sources) inoculated with various strains of MG sampled at day 10 pi	148
4.3.5c.11	Histological lesion scoring of embryonated chicken eggs from Farm B (different sources) inoculated with various strains of MG sampled at day 13 pi	148



4.3.5c.12	Histological lesion scoring of embryonated chicken eggs from Farm B (different sources) inoculated with various strains of MG sampled at day 15 pi	149
4.3.5c.13	Histological lesion scoring of embryonated chicken eggs from Farm C (different sources) inoculated with various strains of MG sampled at day 7 pi	150
4.3.5c.14	Histological lesion scoring of embryonated chicken eggs from Farm C (different sources) inoculated with various strains of MG sampled at day 10 pi	151
4.3.5c.15	Histological lesion scoring of embryonated chicken eggs from Farm C (different sources) inoculated with various strains of MG sampled at day 13 pi	151
4.3.5c.16	Histological lesion scoring of embryonated chicken eggs from Farm C (different sources) inoculated with various strains of MG sampled at day 15 pi	152



LISTS OF PLATES

Plate		Page
3.2.1	Choanal cleft swab sampling	36
3.2.2	Trachea swab sampling	36
3.2.2a	Pipped embryos	37
3.2.2b	Poor quality chicks	39
3.2.2c	Normal chicks	39
3.2.2d	Grading of air sacs lesions	40
3.5.1a	Embryo shows clear and translucent air sac	50
3.5.1b	Embryo shows airsac lesion severity of 1	50
3.5.1c	Embryo shows airsac lesion severity of 2	50
3.5.1d	Embryo shows airsac lesion severity of 3	50
3.5.2a	Differences in colony sizes and shapes (40x)	53
3.5.2b	Glucose fermentation test	54
3.5.2c	Hemadsorption test	54
3.5.2d	IFA result evaluation	54
4.3.2.1a.i	SPF embryo from control group shows clear and translucent air sac or severity of 0	113
4.3.2.1a.ii	SPF embryo of MG inoculated group shows severity of 1	114
4.3.2.1b.i	Commercial embryo from control group shows clear and translucent airsac, severity of 0	116
4.3.2.1b.ii	Commercial embryo of MG inoculated group shows severity of 1	116
4.3.2.1b.iii	Commercial embryo of MG inoculated group shows severity of 2	116
4.3.2.1b.iv	Commercial embryo of MG inoculated group shows severity of 3	116
4.3.2.2a	SPF embryo from MG infected group (I) shows green liver when compared to control embryo (N).	118
4.3.2.2b	Commercial embryo from MG infected group (I) show green liver when compared to control embryo (N)	119
4.3.2.3a	SPF embryo from MG infected group (I) shows head edema and pale when compared to control embryo (N)	120
4.3.2.3b	SPF embryo from MG infected group (I) shows curled toes when compared to control embryo (N)	121
4.3.2.3c	SPF embryo from MG infected group (I) shows dwarfing when compared to control embryo (N)	121
4.3.2.3d	SPF embryo from MG infected group (N) shows pale and slightly enlarged liver when compared to control embryo (N)	121



4.3.2.3e	SPF embryo from MG infected group (I) shows slightly enlarged spleen and pale when compared to control embryo (N)	122
4.3.2.3f	Commercial embryo from MG infected group (I) shows curled toes when compared to control embryo (N)	122
4.3.2.3g	Commercial embryo from MG infected group (I) shows dwarfing when compared to control embryo (N)	123
4.3.2.3h	Commercial embryo from MG infected group (I) shows white spotted liver when compared to control embryo (N)	123
4.3.2.3i	Commercial embryo from MG infected group (I) shows pale and slightly enlarged liver when compared to control embryo (N)	123
4.3.3a	SPF: (A) Pipped embryos (arrows), (B) Dead-in-shell and (C) Poor quality chick	124
4.3.3b	Commercial: (A) Normal chicks, (B) Poor quality chicks and (C) Pipped embryo	125



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
<i>E. coli</i>	<i>Escherichia coli</i>
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	<i>Mycoplasma gallisepticum</i>
mg	milligram
mgc2	Cytadhesin membrane protein
MgCl ₂	Magnesium chloride
ml	milliliter
mm	millimeter
mM	milli Molar
MS	<i>Mycoplasma synoviae</i>
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