การวิเคราะห์ยีนก่อโรคและความรุนแรงในการก่อโรคของเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* ที่แยกได้ในประเทศไทย

นางพัชราภรณ์ ขำพิมพ์

วิทยานิพนธ์นี้ เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2555

VIRULENCE GENE ANALYSIS AND PATHOGENICITY OF THAI ISOLATED *MYCOPLASMA GALLISEPTICUM*

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A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	VIRULENCE GENE ANALYSIS AND PATHOGENICITY OF THAI ISOLATED *MYCOPLASMA GALLISEPTICUM*
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พัชราภรณ์ ขำพิมพ์: การวิเคราะห์ยีนก่อโรคและความรุนแรงในการก่อโรคของเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม*ที่แยกได้ในประเทศไทย. (VIRULENCE GENE ANALYSIS AND PATHOGENICITY OF THAI ISOLATED *MYCOPLASMA GALLISEPTICUM*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.น.สพ.ดร.สมศักดิ์ ภัคภิญโญ,อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ.น.สพ.ดร.จิโรจ ศศิปรียจันทร์, 57 หน้า.

ยีนก่อโรค 4 ชนิด (ยีน *pvpA, gapA, mgc2* และ *LP* (*MGA_0319*)) ของเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* (เอ็มจี) ที่แยกได้จากภาคกลาง ภาคตะวันออก และภาคตะวันตกของประเทศไทยจำนวน 19 สายเชื้อ รวมทั้งเชื้อเอ็มจีสายพันธุ์อ้างอิงจำนวน 2 สายพันธุ์คือ F (สายพันธุ์ของวัคซีน) และ S6 (ATCC® 15302™) ถูกนำมาวิเคราะห์ในการทดลองที่ 1 เพื่อตรวจสอบหาโปรไฟล์ของยีนก่อโรคโดยวิธี PCR ผลการทดสอบพบความหลากหลายของการตรวจพบยีน *gapA* และ *pvpA* ในสารพันธุกรรม โดยพบผลบวกจำนวน 15 และ 4 สายเชื้อจาก19 สายเชื้อตามลำดับขณะที่ยีน *mgc2* และ *LP* พบผลบวกต่อเชื้อที่ทดสอบทั้งหมด ส่วนสายพันธุ์อ้างอิงทั้ง2 สายพันธุ์ให้ผลบวกต่อยีนทั้ง4 ยีน จากการสังเกตพบว่าเชื้อเอ็มจีทั้ง4 สายเชื้อที่ให้ผลลบต่อยีน *gapA* นั้นต่างก็ให้ผลลบต่อยีน*pvpA* ด้วย โดยพบว่ามีเชื้อเอ็มจี3 สายเชื้อที่แยกได้จากภาคตะวันออกและ 1 สายเชื้อแยกได้จากจากภาคตะวันตก ขณะที่เชื้อเอ็มจีที่แยกได้จากภาคกลางทั้งหมดให้ผลบวกต่อยีน *gapA* ผลการศึกษาความรุนแรงของเชื้อเอ็มจีที่แยกได้ในประเทศไทยจำนวน 3 สายเชื้อ(58/46, 31/46 และ 54/46) และเชื้อสายพันธุ์อ้างอิง2 สายพันธุ์เทียบกับกลุ่มควบคุมในไก่ทดลอง(การทดลองที่ 2) และไข่ไก่ฟัก (การทดลองที่ 3) พบว่าให้ผลสอดคล้องกัน โดยเชื้อเอ็มจีสายเชื้อ58/46 (แยกได้จากภาคกลางและให้ผลบวกต่อยีน *gapA* และ *pvpA*) ก่อให้เกิดอาการ ระดับคะแนนรอยโรค และอัตราการตายรุนแรงที่สุด เมื่อเปรียบเทียบกับเชื้อเอ็มจีสายเชื้อ31/46 และ 54/46 (แยกได้จากภาคตะวันออกและให้ผลบวกต่อยีน*gapA* แต่ให้ผลลบต่อยีน *pvpA*) และสายพันธุ์อ้างอิง แสดงว่าเชื้อเอ็มจีสายเชื้อที่ให้ผลลบต่อยีน*pvpA* ก่อให้เกิดความรุนแรงต่ำกว่าสายพันธุ์ที่ให้ผลบวกต่อยีน*pvpA*

ภาควิชา .....อายุรศาสตร์..... ลายมือชื่อนิสิต .....

สาขาวิชา.....อายุรศาสตร์สัตวแพทย์.. ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา...2555.....ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: CHICKENS/ CHICKEN EMBRYONATED EGGS/ *MYCOPLASMA GALLISEPTICUM/ PATHOGENICITY/* VIRULENCE GENE

PACHARAPORN KHUMPIM: VIRULENCE GENE ANALYSIS AND PATHOGENICITY OF THAI ISOLATED *MYCOPLASMA GALLISEPTICUM*. ADVISOR: ASSOC. PROF. SOMSAK PAKPINYO, Ph.D., CO-ADVISOR: PROF. JIROJ SASIPREEYAJAN, Ph.D., 57 pp.

The four virulence genes (*pvpA, gapA, mgc2* and *LP* (*MGA_0319*)) were analyzed from 19 isolates of *Mycoplasma gallisepticum* (MG) from central, eastern and western parts of Thailand and 2 reference strains including F (vaccine strain) and S6 (ATCC® 15302™) by PCR (exp. 1). Results revealed the variation in *gapA* and *pvpA* genes existence. The *gapA* and *pvpA* genes were found in 15 and 4 of 19 isolates, respectively. Whereas, *mgc2* and *LP* genes were detected in all isolates. PCR results of 2 reference strains were positive for all 4 genes. Interestingly, all 4 *gapA*-negative isolates also performed *pvpA*-negative PCR results. Additionally, 3 isolates were obtained from the eastern and 1 from western parts; on the other hand, all MG isolates from the central part showed *gapA*-positive results. The pathogenicity study of 3 Thai MG isolates (58/46, 31/46 and 54/46) and 2 reference strains compared with the control group were determined in chickens (exp. 2) and chicken embryonated eggs (CEE) (exp. 3). The results showed that the pathogenicity study in chickens and CEE were similar. The 58/46 isolate (from the central part, *gapA and pvpA*-positive) caused the most severe clinical signs, lesion scores and mortality compared with 31/46 and 54/46 (from eastern part, *gapA*-positive*, pvpA*-negative) and reference strains did. This experiment suggested that *pvpA*-negative isolates produced the lower virulent than *pvpA*-positive isolates.

Department: .....Veterinary Medicine... Student's Signature.....

Field of Study: ...Veterinary Medicine... Advisor's Signature.....

Academic Year: ... 2012..... Co-advisor's Signature.....

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AS	airsac score
CDSs	coding DNA sequences
CEE	chicken embryonated egg
CEF	chicken embryo fibroblast
CFU	colony forming unit
CrmA	cytadherence-related molecule A
ELISA	enzyme linked immunosorbent assay
GTS	gene-targeted sequencing
HI	hemagglutination inhibition test
kb	kilobase (1 kb = 1,000 base pairs)
kDa	kilodalton
LP	lipoprotein
MG	*Mycoplasma gallisepticum*
MIC	minimum inhibitory concentration
MslA	*Mycoplasma*-specific lipoprotein A
OIE	Office International des Epizooties (World Organization for Animal Health)
PCR	polymerase chain reaction
RAPD	random amplification of polymorphic DNA
SPA	serum plate agglutination test
S.D.	standard deviation
TS	tracheal score
$\bar{x}$	mean

CHAPTER I
INTRODUCTION

Background and Rationale

Mycoplasma gallisepticum (MG) infection is the important respiratory disease of chicken, called as chronic respiratory disease (CRD) or airsac disease, which causes vary economic losses including high mortality, high culling rate, low producitivity of both egg and body weight, and also increases diagnosis, vaccination and medication costs. Moreover, MG infection is related to carcass condemnation from under-targeted body weight and lesions of airsacs. In case of secondary infection, complicated with infectious bronchitis or Newcastle disease or *Escherichia coli*, the lesions would be more severe and widely distributed to other organs. In this case, slaughter house cannot catch those carcasses into the process, which results in additionally economic losses. Although MG prevention is intensively promoted in poultry farm nowadays, the disease still frequently occurs in farm worldwide, including Thailand. Supplement measures should be launched for more effective MG prevention and control (Evans et al., 2005; Sasipreeyajan, 2007).

Clinical signs of MG infection in chicken include respiratory rales, coughing, sneezing, nasal discharge and conjunctivitis. Disease can transmit to normal chickens by aerosol and transovarian routes. Other important characteristics are that slowly develops clinical sign and has a long course of disease (Sasipreeyajan, 1989; Ley, 2008). Once outbreak occurs, disease will be rapidly spread within the flock then formed chronic disease which is difficult to be controlled by antibiotics. Chronic MG infection occurring within the flock implies that chicken immunity cannot completely clear MG organisms (Levisohn et al., 1995; Glew et al., 2000).

In the past, scientists believed that MG was an extracellullar organism; however, recently revealed that MG is an intracellullar organism (Winner et al., 2000). That is the reason why MG can resist to host immunity and antibiotics (Winner et al., 2000; Nascimento et al., 2005). After entering into the host, MG primarily grow in the respiratory tract, but other organs such as reproductive tract, brain and eyes are also targeted (Ley, 2008). The important mechanism in pathogenesis of MG is

"cytadherence" with host epithelial cells surface before cell proliferation (Nascimento et al., 2005; Ley, 2008). The cytadhesion related to the variable cytadhesin protein such as MGC2 cytadhesin protein (Hnatow et al., 1998), GapA cytadhesin protein (Goh et al., 1998), CrmA putative cytadhesin related molecule (Papazisi et al., 2002) and PvpA putative variable cytadhesin (Boguslovsky et al, 2000). Those molecules are required to work together in cytadherence process, so lacking of some proteins results in loss of infectivity (Razin and Jacob, 1992). Genes related to cytadherence include *mgc2, pvpA, gapA* and *crmA* genes (Goh et al., 1998; Hnatow et al., 1998; Boguslovsky et al, 2000; Papazisi et al., 2002).

Because MG lacks a peptidoglycan cell wall and can easily change itself suggesting that antigenic variation of surface antigen always occurs (Razin et al., 1998). These changes possibly support the entering of MG into the host cells and the MG survival for a long time in the host cells (Papazisi et al., 2003). The related genes, which always genetically change by time, include *pMGAs* (hemagglutinins), *mgc1*, *mgc2* and *pvpA* (Papazisi et al., 2003; Nascimento et al., 2005).

There are several diagnostic techniques for MG such as histopathology, microbiology (isolation), serology including serum plate agglutination (SPA) test, enzyme linked immunosorbent assay (ELISA), hemagglutination inhibition (HI) test and molecular biology (polymerase chain reaction (PCR)). MG isolation is time consuming which may last longer than 21 days, thus the more convenient methods used for MG diagnosis are serology tests and PCR (OIE, 2004). Serology tests are significantly beneficial tools for flock monitoring control program. SPA test is very important for detection in early MG infection. However, ELISA is the test of choice for MG serology as ELISA is more specific than SPA and more sensitive than HI test. The PCR test is also the valuable tool for MG infection diagnosis because it is a rapid and sensitive method to detect the DNA of organism, especially in tracheal swab samples collected from field, compared with isolation technique (OIE, 2004).

Control and prevention of MG in the farm consist of strict biosecurity, biosurveillance and vaccination, especially in layer chickens and parent stocks. Vaccines have been developed including live to other alternative vaccines such as bacterin and subunit vaccine, but the vaccine efficacy in disease protection is still needed to be further studied and improved (Evans et al., 2005).

In Thailand, MG was studied in biopathology (Pakpinyo, 2005; Pakpinyo et al., 2011), diagnostic laboratory technique development (Pakpinyo et al., 2006), disease epidemiology (Pakpinyo et al., 2007), antibiotics efficacy for MG treatment (Pakpinyo et al., 2008), and genetic variation using random amplification of polymorphic DNA (RAPD) (Pakpinyo and Sasipreeyajan, 2007). However; the analysis of Thai MG virulence genes has not been reported and is the interesting issue that whether various pathogenic Thai MG field strains have differences in virulence gene profile.

Objectives of present study

The aims of this study were to analyze 4 virulence genes (*pvpA, gapA, mgc2* and LP (*MGA_0319*) gene) of Thai MG isolated by using PCR (Ferguson et al., 2005) and to evaluate the pathogenicity of 3 geographically different isolates of Thai MG isolated compared with two reference strains (F and S6) in chicken embryonated eggs and experimental chickens.

Expected output

The expected output which obtains from this study is to know the virulence gene profile and pathogenicity of Thai MG field isolates from each parts of Thailand which is beneficial in control and prevention of MG in each region and also useful for the development of MG vaccine in the future.

CHAPTER II
LITERATURE REVIEW

Biology of *Mycoplasma gallisepticum*

Mycoplasma gallisepticum (MG) is classified in the class Mollicutes, order Mycoplasmatales, family Mycoplasmataceae and genus *Mycoplasma* (OIE, 2004). Mycoplasmas are distinguished from other bacteria by their small size and total lack of a cell wall, that is the reason why their class named *Mollicutes* (*mollis* is soft and *cutis* means skin, in Latin) (Razin et al., 1998; Ley, 2008). Lack of cell wall is the underlying reason for the "fried egg" colony when it grows on solid media. In addition, mycoplasmas are completely resistant to the action of antibiotics that interact with cell wall-related proteins due to the absence of cell wall material and those associated proteins. Mycoplasmas have a tiny genomes size with 580 to 1,350 kb, or approximately only one-sixth the size of the *Escherichia coli* genome, and their G + C content is very low as 23-40 % (Rosenbusch, 1994; Papazisi et al., 2003).

Fraser et al. (1995) launched the first report of complete genome sequencing of Mycoplasmas, as *Mycoplasma genitalium* (580 kb), which possesses the smallest genomes of free-living organisms. Since this initial report, the genomes of several additional mycoplasmas have been sequenced, *Mycoplasma pneumoniae* (816 kb; Himmelreich et al., 1996; Dandekar et al., 2000), *Ureaplasma urealyticum* (752 kb; Glass et al., 2000), *Mycoplasma penetrans* (1,358 kb; Sasaki et al., 2002), *Mycoplasma pulmonis* (964 kb; Chambaud et al., 2001), *Mycoplasma suis* (742 kb; Guimaraes et al., 2011), *Mycoplasma hyorhinis* strain SK76 (836 kb; Goodison et al, 2013), including avian Mycoplasma, *Mycoplasma gallisepticum* strain R_{low} (996 kb; Papazisi et al., 2003). *Mycoplasma gallisepticum* strain R_{low} composed of 996,422 bp with overall G + C content of 31 % (Papazisi et al., 2003). The complete genome features of *Mycoplasma gallisepticum* strain R_{low} are shown in Figure 1.

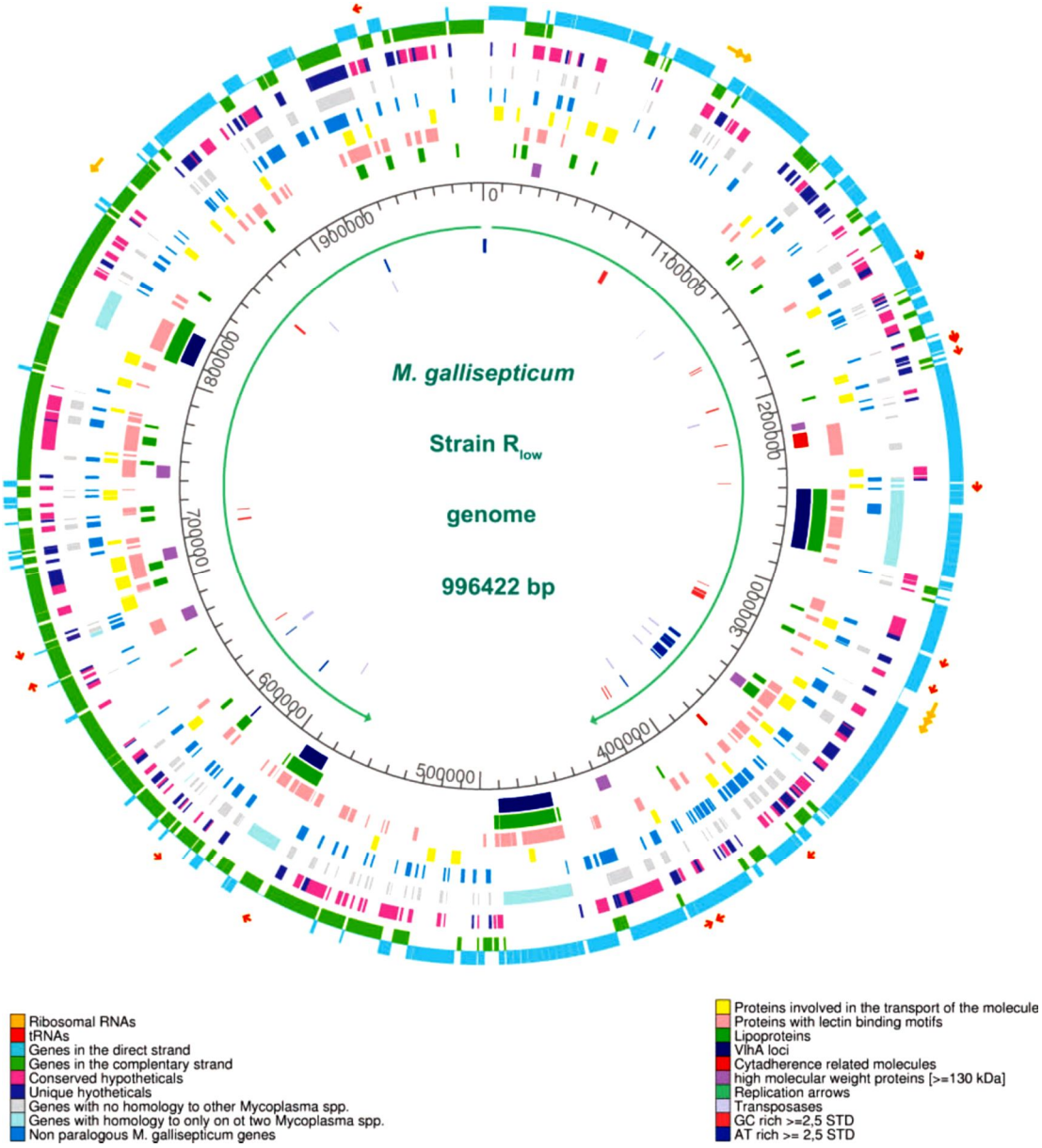


Figure 1. Circular representation of the *M. gallisepticum* strain R_{low} genome. This figure was generated using GenVision (DNAStar).

(Papazisi et al., 2003)

Studies of MG pathogenicity

The studies of MG pathogenicity performed both *In vitro* in cell culture (Winner et al., 2000) and chicken tracheal organ culture (Cherry and Taylor-Robinson, 1970, 1971), and *In vivo* in experimental chickens or turkeys (Varley and Jordan, 1978[a, b]; Levisohn et al., 1986; Yagihashi et al., 1988; Much et al., 2002) and chicken embryonated eggs (Levisohn et al., 1985). These reports demonstrated the differences in MG pathogenicity of the different MG isolates in many countries. In Thailand, there were a few reports of various Thai MG isolates. Pakpinyo (2005) studied on pathogenicity of MG isolated from the broilers in Thailand compared with S6 strain and revealed that Thai MG isolated had more pathogenicity than S6 strain. Then, Pakpinyo and Sasipreeyajan (2007) classified field isolates of Thai MG into 5 groups and evaluated minimum inhibitory concentration (MIC) of some antibiotics of each group. The results showed that doxycycline and tiamulin had the lowest MIC level in all 5 groups. Furthermore, Pakpinyo et al. (2008) tested the efficacy of tilmicosin in broilers compared with S6 strain and found that tilmicosin could decrease morbidity and lesion severity in MG inoculated chickens. In 2011, Pakpinyo et al. studied Thai MG pathogenicity in chicken embryonated eggs compared with MG vaccine strains.

Studies of MG genetics and virulence genes

Numerous researches on MG genetics have been developed after Papazisi et al. (2003) demonstrated whole genome of R_{low} strain. From this report, MG R_{low} strain characteristics were revealed such as the size of 996,422 bp with 31 mol% G+C contents and 742 coding DNA sequences (CDSs), which accounted as 91% of coding density. Function has been assigned to 469 of the CDSs, while 150 encode conserved hypothetical proteins and 123 remaining as unique hypothetical proteins (Papazisi et al., 2003). Several genes were used for genetic variation studies such as *16S rRNA*, *mgc2*, *mgc1 (gapA)*, *pvpA* and surface lipoprotein gene (*LP*) (Furguson et al., 2005; Szczepanek et al., 2010). Ferguson et al. (2005) studied genetic variation of 4 genes (*pvpA, gapA, mgc2* and *LP* (*MGA_0319*) gene), which related with their cell surface and

pathogenicity, of 67 American, Israeli and Australian MG isolates including 10 reference strains using random amplification of polymorphic DNA (RAPD), gene-targeted sequencing (GTS) and phylogenetic analysis. The results showed genetic variation with insertion and/or deletion of genes and also found the epidemiological association with disease outbreak in those areas. Analysis of MG R_{high} strain, attenuated derivative of R_{low}, revealed that 64 loci genomic changes occurred compared to virulent R_{low} strain. This indicated that the genomic changes ocurred in R_{high} strain resulted in attenuation of the organism (Szczepanek, 2009).

Virulence genes analysis of MG were mostly studied on genes related to surface molecule of cell membrane, which plays a role in cytadherence with the host cell during infection (Papazisi et al., 2002). MG cell membrane consists of approximately 200 polypeptide chains which play a role in surface antigenic variation, cytadherence with the host cell, motility and dietary transportation. The important proteins of MG which function in host immune response are adhesions or hemagglutinins. These proteins will bind to receptor site on host epithelium, result in MG colonization and then infection will progress from this location (Ley, 2008). The genes related to former processes are further described.

1. ***pMGA* genes or *vlhA* genes**

Papazisi et al. (2003) renamed the pMGA genes as *vlhA* genes to concordance with current nomenclature. These genes encode the hemagglutinins (Ley, 2008). The *vlhA* gene family has 43 genes distributed in 5 multigene loci (8, 2, 9, 12 and 12 genes) around the chromosome, constituting a total of 103 kb or 10.4% of the genome (Papazisi et al., 2003). This *vlhA* gene family also take the role in encoding of some membrane proteins such as P52 and P67 MG-specific lipoproteins (Jan et al., 2001).

The variation of *vlhA* genes would affect the pathogenicity. Such a case of F strain, which at least 16 genes deleted including *vlhA* gene family and there were the changes of some base sequences especially in GAA repeat. These changes resulted F strain is 35 kb smaller than R_{low} strain, and also has less severity (Glew et al., 2000; Szczepanek, 2009).

2. *gapA* gene or *mgc1* gene

The *gapA* cytadhesin gene also referred as *mgc1* (Goh et al., 1998). The 2,895 bp *gapA* gene has the role in encoding of the 105 kDa GapA, the important protein known as primary cytadhesin (Goh et al., 1998; Mudahi-Orenstein et al., 2003). The *gapA* gene works together with other genes such as *CrmA* to synthesize the cytadherence-related protein (Papazisi et al., 2002; Ley, 2008). Goh et al. (1998) proved the role of *gapA* gene in host cell cytadherence using anti-GapA Fab fragments to inhibit the activity. Results revealed that anti-GapA Fab fragments could inhibit the MG attachment to tracheal epithelium cells approximately 64% (Goh et al., 1998). There was the report described that some avirulent MG strains were lacking of the GapA protein (Papazisi et al., 2002).

3. *pvpA* gene

The *pvpA* gene encodes the accessory adhesions protein family such as PvpA and p67a which is associated to cytadherence and immunogenicity (Ogle et al., 1992; Goh et al., 1998; Hnatow et al., 1998; Boguslavsky et al., 2000). PvpA cytadhesin is a non-lipid integral membrane, surface-exposed immunogenic protein. The surface-exposed C terminus of PvpA protein consists of a high proline content by 28% and contains identical direct repeat sequences consisting of 52 amino acids each, called DR-1 and DR-2 (Boguslavsky et al., 2000; Liu et al., 2001). The size of this gene depends on the MG strain, as a result of deletion of the segment encoding the C-terminal region of PvpA protein (Figure 2) (Boguslavsky et al., 2000). Interestingly, MG *pvpA* gene has been studied for epidemiological researches due to their genomic variation (Boguslavsky et al., 2000; Liu et al., 2001; Papazisi et al., 2003; Ferguson et al., 2005).

Although PvpA is a phase-variable protein recognized by the chicken immune system (Yogev et al., 1994; Levisohn et al., 1995; Liu et al., 2001), the immune recognition of PvpA is weak, this involve the lack of modulation of host immune system (Levisohn et al., 1995). Recently, MG PvpA cytadhesin has been cloned in *Escherichia*

coli and used as a species-specific recombinant antigen to develop an individual rapid test for MG infections screening in the field (Büyüktanır et al., 2010).

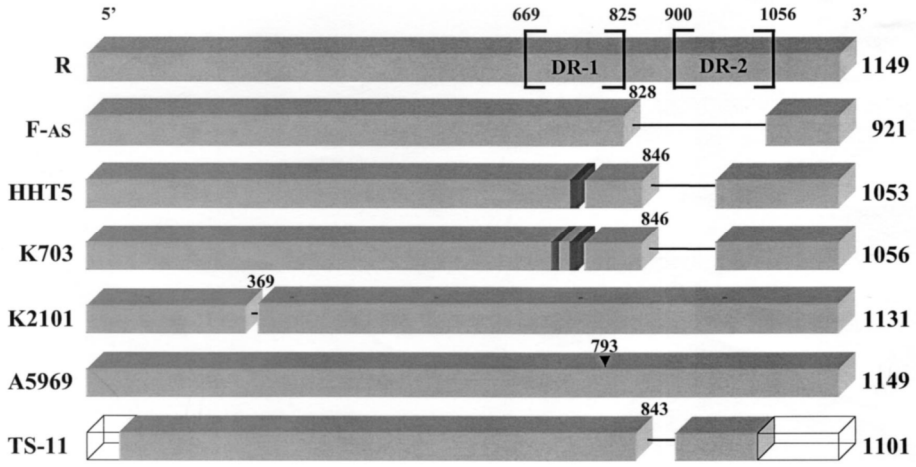


Figure 2. Size variation and deletions within the C terminus-encoding region of the *pvpA* gene. Gaps within the *pvpA* genes represent various types of deletions in comparison to strain R. Small dark rectangles indicate nucleotide sequences within the *pvpA* gene of strain HHT5 and K703 which are not present in the R strain. Small dark rectangles indicate nucleotide sequences within the *pvpA* gene of strain HHT5 and K703 which are not present in the R strain. The numbers at the beginning of each deletion indicate the nucleotide position. Open rectangles in the vaccine strain ts-11 represent regions which were not sequenced.

(Boguslavsky et al., 2000)

4. *crmA* gene

The *crmA* gene encodes 116 kDa CrmA (cytadherence-related molecule A) protein, which relates to cell attachment (Mudahi-Orenstein et al., 2003). Papazisi et al. (2002) studied this gene in cell culture using MRC-5 cell and found that *crmA* gene had to work together with *gapA* gene in host cell attachment (Papazisi et al., 2000, 2002). Winner et al. (2003) also studied this gene and *gapA* gene on the ability of the cell attachment using sheep red blood cells. The results showed that GapA and CrmA protien expression disappeared in non-hemadsorption clones of MG (Winner et al.,

2003). The *crmA* gene located downstream of the *gapA* gene as part of the same operon (Figure 3) (Mudahi-Orenstein et al, 2003). The *crmA* and *gapA* gene coexpressed in MG R_{low} strain which is pathogenic strain but they disappear in non virulent strain such as R_{high} (passage 164) strain (Papazisi et al., 2000; Mudahi-Orenstein et al., 2003). The sequence analysis reveals that the GapA and CrmA cytoplasmic tails are similar in amino acid sequencing and play as the role in DNA binding and protein-protein interactions. The *crmA* and *gapA* gene also coexpressed in regulation of other MG cytadherence-related genes (Papazisi et al., 2002).

5. *mgc2* gene

The *mgc2* gene encodes a 32.6-kDa MGC2 protein which locates at the end of cytadhesin molecule and works with GapA/MGC1 protein in cytadherence to host cell (Figure 3) (Boguslavsky et al., 2000; Mudahi-Orenstein et al., 2003). One study using rabbit anti-MGC2 antiserum could inhibit the host cell attachment approximately 37-48%. The results suggested that MGC2 requires some other factors to support cytadherence. Moreover, chicken embryo fibroblast (CEF) cells incubated with Neuraminidase showed 50-65% inhibition in host cell attachment (Hnatow et al., 1998).

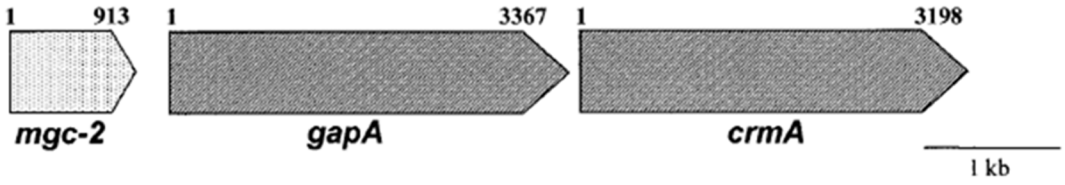


Figure 3. The size, locations and directions of the *mgc-2*, *gapA*, and *crmA* genes of *Mycoplasma gallisepticum*

(Mudahi-Orenstein et al., 2003)

6. *hmw3* gene

The *hmw3* gene encodes HMW3 protein, cytadherence-accessory protein or accessory cytadhesin, which functions indirectly in attachment to host cell and enhances the potential of cytadhernce process (Ogle et al., 1992).

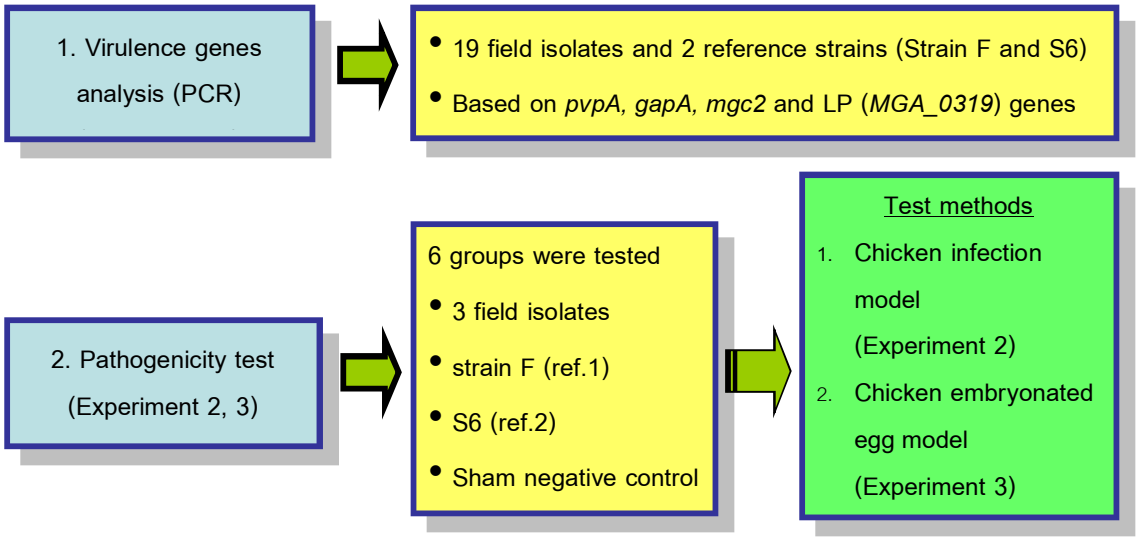
7. Surface lipoprotein (LP) encoding gene

MG has 80 genes encoding LP including *MGA_0319* gene encoding the conserved hypothetical lipoprotein, *MGA_0674* gene encoding *Mycoplasma*-specific lipoprotein A (MslA). Szczepanek et al. (2010) found that MslA protein related to antigenicity of MG cell surface and pathogenicity. The MslA protein revealed to lower expression in attenuated strain (Furguson et al., 2005; Szczepanek et al., 2010).

In the literature review, virulence genes of MG have been individually studied. The whole virulence gene profile of MG has not been reported, but in other bacteria Schierack et al. (2006) determined virulence gene profile of *E. coli* in swine. This study revealed that the virulence genes could be found in *E. coli* isolated from the healthy pigs. This suggested that the virulence genes existence in organism may not associated with their pathogenicity (Schierack et al., 2006).

CHAPTER III
MATERIALS AND METHODS

Conceptual framework of this study



Materials and Methods

1. *M. gallisepticum* isolates:

Twenty one MG isolates (by Somsak Pakpinyo, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University) were used. Nineteen of these isolates obtained from broiler breeder, broiler and layer farms of different parts of Thailand between 2007-2008 (Table 1). All isolates obtained from farms were cultured and stored in Frey's broth medium at -80^{0}C. All frozen isolates were re-propagated in Frey's medium until the broth color changed from red to orange or yellow before used in the experiments. Two reference strains were used in this experiment. MG S6 strain (ATCC® 15302™) was obtained from the American Type Culture Collection, and MG F (Intervet Schering-Plough Animal Health, Thailand) vaccine strain was provided by local distributors.

Table 1. Source of each isolate using in virulence genes analysis

No.	Strain		Source
1	1/45	1.3P	Central part
2	1/45	11.3P	Central part
3	1/46	3.3P	Central part
4**	2/46	3.2P	Eastern part
5	7/46	1.2P	Eastern part
6	8/46	2.2P	Eastern part
7	20/52	6.1P	Western part
8*	31/46	1.2P	Eastern part
9	32/46	15.1P	Eastern part
10	33/46	2.3P	Eastern part
11	34/46	1.1P	Eastern part
12	50/46	4.2P	Central part
13	51/46	13.2P	Central part
14	51/46	13.3P	Central part
15	53/46	11.2P	Eastern part
16*	54/46	15.2P	Eastern part
17	55/46	5.3P	Eastern part
18	55/46	7.3P	Eastern part
19	57/46	13.2P	Eastern part
20*	58/46	8.2P	Central part
21*	S6		ATCC® 15302™
22*	F		Intervet Schering Plough (F vax)
23	+ ve		Intervet Schering Plough (F vax)
24	- ve		Negative control

* means the MG isolates using in pathogenicity tests (Experiment 2 and 3)

** means the isolate that is not MG

2. Experiment 1 Virulence gene analysis

2.1) Polymerase chain reaction (PCR)

2.1.1) MG DNA was extracted from 150-250 µl MG cultured Frey's broth and amplified by using the previously described protocol (Lauerman, 1998). Briefly,

2.1.1.1) Pelleted MG cultured Frey's broth by centrifugation at 15,000xg for 6 minutes

2.1.1.2) Washed with distilled water and discarded the supernatant

2.1.1.3) Resuspended with distilled water

2.1.1.4) Boiled for 10 minutes

2.1.1.5) Quickly placed on ice for 10 minutes

2.1.1.6) Centrifuged at 15,000xg for 2 minutes

2.1.1.7) The supernatant containing DNA was collected and stored at -20°C until used for further PCR.

2.1.2) Prepared 45 µl PCR mixture of each MG DNA sample. Primers of 4 virulence genes (*LP* (MGA_0319), *gapA*, *pvpA* and *mgc2* gene) used in virulence gene analysis (Ferguson et al., 2005) were shown in Table 2.

2.1.3) Add 5 µl DNA template for each reaction.

2.1.4) The amplification conditions for all 4 genes were 95^0C for 5 min, and 35 cycles of 95^0C for 30 s, 58^0C for 30 s and 72^0C for 45 s, and 72^0C for 5 min.

2.1.5) Each reaction was performed concurrently with the F strain as a positive and distilled water as a negative control.

2.1.6) The expected amplicon were 590, 332, 702 and 824 bp, respectively.

2.1.7) DNA fragments were separated by gel electrophoresis in 2% agarose gel, bands were visualized by UV transilluminator after ethidium bromide staining, and then photographed.

2.1.8) Recorded the number of positive results from 4 virulence genes of all 21 isolates.

Table 2. Details of primers and PCR products used in virulence gene analysis

Primer	Sequence (5'→ 3')	Location		PCR Product size
		Genome CDS	nt position[2]	
lp 1F	CCAGGCATTTAAAAATCCCAAAGACC	MGA_0319 C[1]	906–931	590
lp 1R	GGATCCCATCTCGACCACGAGAAAA		1471–1495	
gapA 3F	TTCTAGCGCTTTAGCCCTAAACCC	MGA_0934	2601–2624	332
gapA 4R	CTTGTGGAACAGCAACGTATTCGC		2909–2932	
pvpA 3F	GCCAMTCCAACTCAACAAGCTGA	MGAL_0258_0256 C[1]	545–567	702
pvpA 4R	GGACGTSGTCCTGGCTGGTTAGC		1224–1246	
mgc2 1F	GCTTTGTGTTCTCGGGTGCTA	MGA_0932	53–73	824
mgc2 1R	CGGTGGAAAACCAGCTCTTG		857–876	

[1] C means complement of the *M. gallisepticum* R_{low} genome CDS

[2] Nucleotide positions of primers based on *M. gallisepticum* R_{low} genome CDS (Ferguson et al., 2005)

2.2) MG inoculum preparation for experiments 2 and 3

2.2.1) 0.2 ml of MG cultures in Frey's broth containing with 15% swine serum (Kleven, 1998) and keeped at -80°C were propagated in 10 ml Frey's broth and then incubated at 37 °C until the color of broth changed to orange-yellow.

2.2.2) Stored in 1 ml aliquots at -80°C before used.

2.2.3) One of aliquot from 2.2.2 was counted for MG colony (colony forming unit (CFU) / per ml).

2.2.4) Diluted in 10-fold dilution with Frey's broth containing 15% swine serum, then cultured each dilution on Frey's agar plate containing 15% swine serum and incubated at 37 °C for 7 days.

2.2.5) Counted MG colony and calculated the concentration in CFU/ml and recorded as the representative of remaining aliquots from 2.2.2

2.2.6) At day of challenge, each aliquot from 2.2.2 was prepared to concentration of $1.0x10^7$CFU/ml using for the experiments in chicken or embryonated egg model and consequently determined the concentration of inoculum by MG colony count.

3. Experiment 2 Pathogenicity study in chicken infection model

3.1) One hundred and fifty six, one day-old chicks were obtained from MG and MS free breeder farm raised together until 21 days of age.

3.2) At 21 days of age, 30 chickens were randomly sampled for MG status before starting the experiment. MG status included blood collection for MG ELISA test (Synbiotics, San Diego, CA), necropsy for evaluation of lesion scores of gross thoracic airsacs (AS) (Kleven et al., 1972) (Appendix A) and histopathologic trachea (TS) (Yagihashi and Tajima, 1986) (Appendix B), and airsac swab (3 swabs were pooled as 1 sample) for MG PCR detection.

3.3) The remaining chickens were divided into 6 groups of 21 birds as showed in table 3. Each group was inoculated MG via intranasal route with 0.1 ml sham negative control or $1.0x10^7$ CFU/ml MG inocula of S6, F, 31/46, 54/46 and 58/46 strain, respectively. Challenged chickens were seperately raised in 70x80x65 cm cage (Figure 4). Each group was kept in separated room with conventional condition and management and fed *ad libitum* until 42 days of age.

3.4) Clinical signs and mortality were daily observed from 21 to 42 days of age. Dead chickens were necropsied for;

3.4.1) Evaluation the lesion scores of AS (Appendix A) and TS (Appendix B),

3.4.2) Swab the airsacs for MG PCR test.

3.5) At 42 days of age, all surviving chickens were collected samples as following:

3.5.1) Bleed to collect serum for MG ELISA (Synbiotics, San Diego, CA). test,

3.5.2) Euthanized, necropsy to observe and blindly evaluate lesion scores of AS (Appendix A) and TS (Appendix B) (Figures 5 and 6),

3.5.3) Swab the airsacs for MG PCR test based on *mgc2* gene (Figure 7).

Table 3 Details of MG inoculation in each experimental group at 21 days of age.

Group	Inoculation detail
1	Inoculated with 0.1 ml Frey's broth (intranasal route)
2	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***MG-S6*** strain in Frey's broth (intranasal route)
3	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***MG-F*** strain in Frey's broth (intranasal route)
4	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***Thai MG (31/46)*** isolate in Frey's broth (intranasal route)
5	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***Thai MG (54/46)*** isolate in Frey's broth (intranasal route)
6	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***Thai MG (58/46)*** isolate in Frey's broth (intranasal route)

4. <u>Experiment 3</u> **Pathogenicity study in chicken embryonated egg model**

4.1) MG- and MS-free 8 day-old chicken embryonated eggs were divided into 6 groups of 15 embryonated eggs (Table 4). Eggs were inoculated via yolk sac route (Appendix C) with 0.1 ml sham negative control or $1.0x10^7$ CFU/ml MG inocula as shown in Table 4.

4.2) The inoculated eggs were incubated and embryonic death was observed twice daily by candling.

4.2.1) Dead embryos were collected for:

(except embryonic death during 24 hours post inoculation period was discarded.)

Figure 4. Raising condition of challenged chickens. Chickens were seperately raised in 70x80x65 cm cage in seperatd room until 42 days of age.

Figure 5. Lesion observation, scoring and sample collection at terminated day. At 42 days of age, chickens were euthanized and necropsied for evaluation of airsac score and other related lesions.

Figure 6. Tracheal sample collection. Trachea of chickens were collected and fixed in 10% formalin for histopathologic trachea.

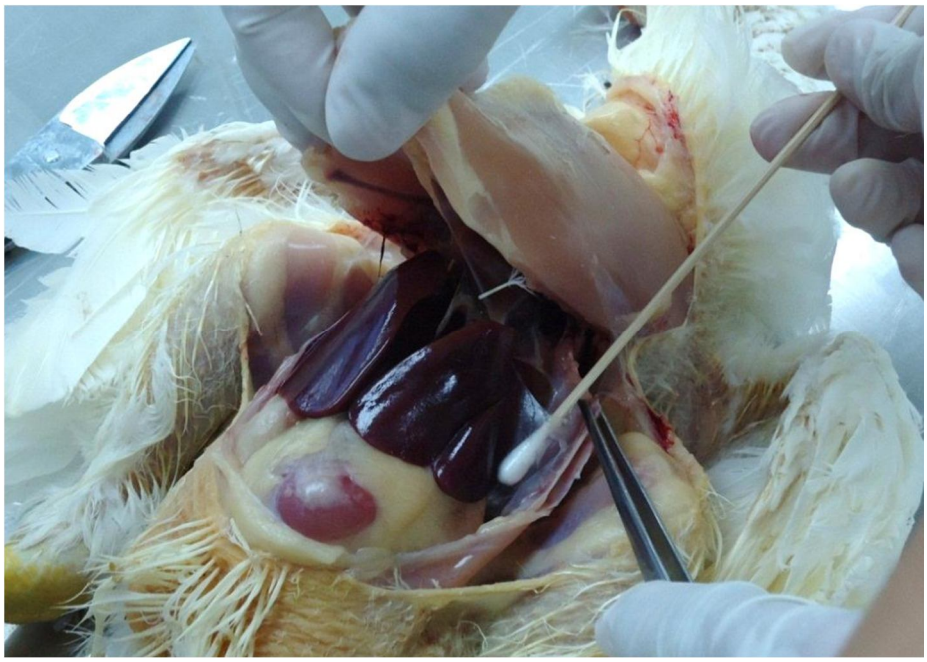


Figure 7. Thoracic airsac sample collection. After evaluating airsac score, airsac swab was performed for MG PCR.

4.2.1.1) Euthanize and necropsy to observe lesions and blindly evaluate lesion scores of AS (see Appendix A) and TS (Appendix B),

4.2.1.2) swab the yolk sacs for MG PCR test based on *mgc2* gene.

4.3) After hatching, all chickens were separately raised in the clean boxes for 7 days (Figure 8). Feed and water were provided *ad libitum*.

Table 4 Details of MG inoculation in each experimental embryonated eggs.

Group	Inoculation detail
1	Inoculated with 0.1 ml Frey's broth (via yolk sac route)
2	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***MG-S6*** strain in Frey's broth (via yolk sac route)
3	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***MG-F*** strain in Frey's broth (via yolk sac route)
4	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***Thai MG (31/46)*** isolate in Frey's broth (via yolk sac route)
5	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***Thai MG (54/46)*** isolate in Frey's broth (via yolk sac route)
6	Inoculated with 0.1 ml of $1.0x10^7$ CFU/ml ***Thai MG (58/46)*** isolate in Frey's broth (via yolk sac route)

4.4) The clinical signs (depression, anorexia, rale, cough, nasal discharge, conjunctivitis or keratitis) and mortality of chicks were daily observed (Figure 9).

4.4.1) The dead chicks were necropsied and samples were collected for;

4.4.1.1) Evaluation the lesion scores of AS (Appendix A) and TS (Appendix B),

4.4.1.2) Swab the airsacs or yolk sac for MG PCR test.

4.5) At 7 days of age, all surviving chickens were euthanized for;

4.5.1) Necropsy to observe lesions (Figure 10) and evaluate lesion scores of AS (Appendix A) and TS (Appendix B),

4.5.2) Swab the airsacs or yolk sac (Figures 10 and 11) for MG PCR test.

Figure 8. Raising condition of hatched chickens. After hatching, all chickens were separately raised in the clean boxes for 7 days. Feed and water were provided *ad libitum*.

Figure 9. Clinical signs and mortality observation. The chicks were daily observed for clinical signs (depression, anorexia, rale, cough, nasal discharge, conjunctivitis or keratitis) and mortality.

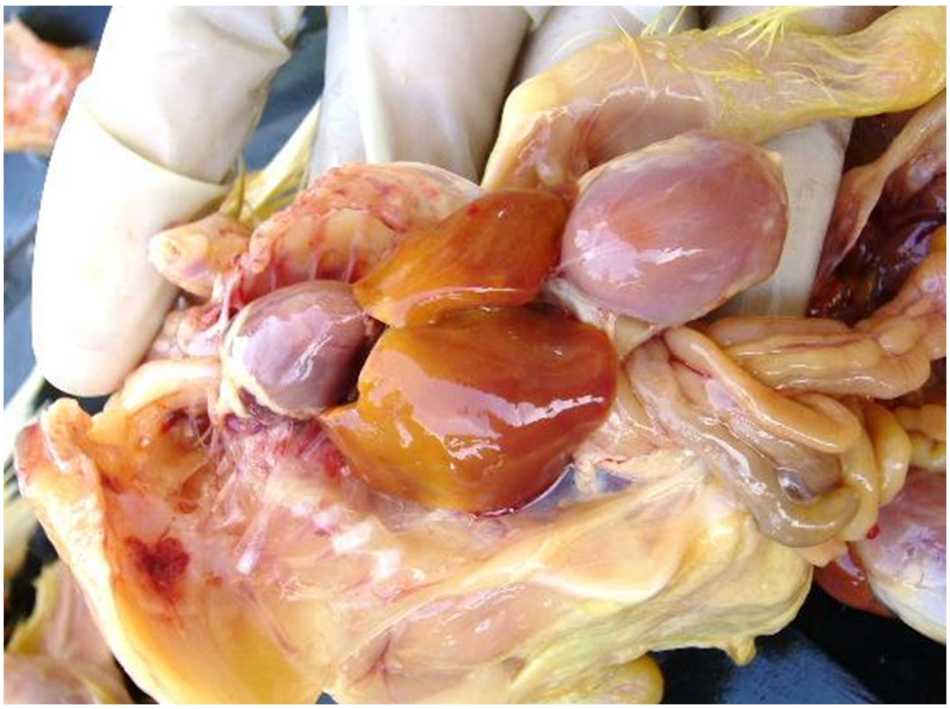


Figure 10. Lesion observation, scoring and sample collection of surviving chickens. At 7 days of age, all surviving chickens were euthanized and necropsied to observe lesions, evaluate lesion scores and collect the samples.

Figure 11. Sample swab in Frey's broth. The airsacs or yolk sac was swabbed and kept in Frey's broth for MG PCR.

Statistical analysis

Numbers of sick and dead chicken were analyzed by using Chi square test. Lesion scores of gross thoracic airsacs and histopathologic trachea were analyzed by using Kruskal-Wallis test and Mann-Whitney U test at 95% confidence level ($p < 0.05$).

CHAPTER IV
RESULTS

1. Virulence gene analysis:

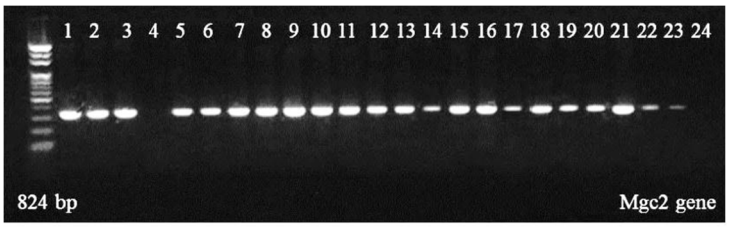
The *mgc2* and *Lp* gene analysis showed that all of Thai MG isolates (19/19) performed positive PCR results. Whereas, *pvpA* gene analysis showed positive PCR results only 4 of 19 Thai MG isolates (8/46 2.2P, 33/46 2.3P, 50/46 4.2P and 51/46 13.2P). The *gapA* gene analysis found 15 of 19 Thai MG isolates (except 7/46 1.2P, 20/52 6.1P, 55/46 5.3P and 57/46 13.2P). The reference strains (S6 and F) showed positive PCR results for all 4 virulence genes (see figure 12 and table 5).

Table 5. PCR results of 4 virulence gene analysis of all 19 Thai MG isolates and reference strains

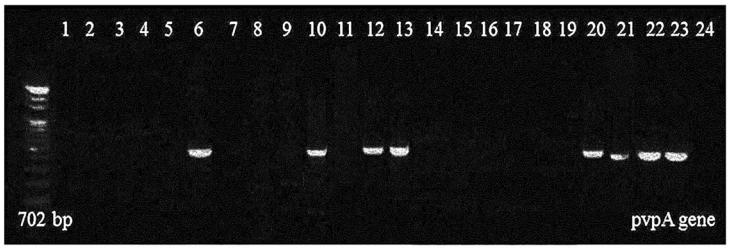
Virulent gene	Number of positive / 19 Thai MG	1.3P 1/45 1	11.3P 1/45 2	3.3P 1/46 3	3.2P 2/46 4**	1.2P 7/46 5	2.2P 8/46 6	6.1P 20/52 7	1.2P 31/46 8*	15.1P 32/46 9	2.3P 33/46 10	1.1P 34/46 11	4.2P 50/46 12	13.2P 51/46 13	13.3P 51/46 14	11.2P 53/46 15	15.2P 54/46 16*	5.3P 55/46 17	7.3P 55/46 18	13.2P 57/46 19	8.2P 58/46 20*	reference S6 21*	reference F 22*	Control + ve 23	Control - ve 24
mgc2	19/19	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
pvpA	4/19	-	-	-	-	-	+	-	-	-	+	-	+	+	-	-	-	-	-	-	+	+	+	+	-
gapA	15/19	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-
Lp	19/19	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

* means the MG isolates using in pathogenicity study (Experiments 2 and 3)

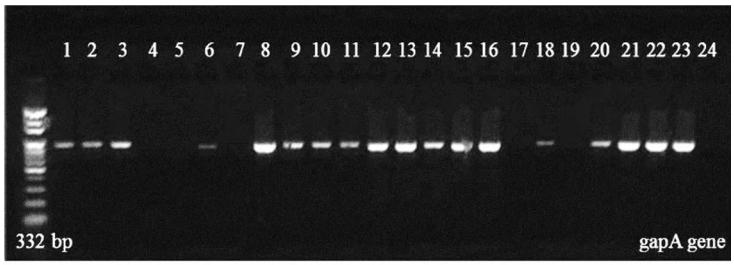
** means the isolate that is not MG



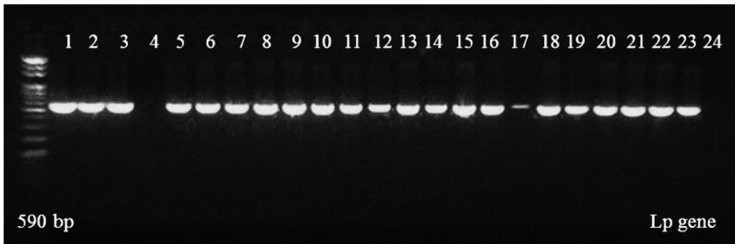
(a)



(b)



(c)



(d)

Lane 1 = marker
Lane2 (#1) = Thai MG (1/45 1.3P)
Lane3 (#2) = Thai MG (1/45 11.3P)
Lane4 (#3) = Thai MG (1/46 3.3P)
Lane5 (#4) = Thai isolate that is not MG (2/46 3.2P)
Lane6 (#5) = Thai MG (7/46 1.2P)
Lane7 (#6) = Thai MG (8/46 2.2P)
Lane8 (#7) = Thai MG (20/52 6.1P)
***Lane9 (#8)** = Thai MG (31/46 1.2P)
Lane10 (#9) = Thai MG (32/46 15.1P)
Lane11 (#10) = Thai MG (33/46 2.3P)
Lane12 (#11) = Thai MG (34/46 1.1P)
Lane13 (#12) = Thai MG (50/46 4.2P)
Lane14 (#13) = Thai MG (51/46 13.2P)
Lane15 (#14) = Thai MG (51/46 13.3P)
Lane16 (#15) = Thai MG (53/46 11.2P)
***Lane17 (#16)** = Thai MG (54/46 15.2P)
Lane18 (#17) = Thai MG (55/46 5.3P)
Lane19 (#18) = Thai MG (55/46 7.3P)
Lane20 (#19) = Thai MG (57/46 13.2P)
***Lane21 (#20)** = Thai MG (58/46 8.2P)
***Lane22 (#21)** = MG-S6 (Ref. strain 1)
***Lane23 (#22)** = MG-F (Ref. strain 2) (Fvax vaccine)
Lane24 (#23) = positive control (Fvax vaccine)
Lane 25 (#24) = negative control (distilled water)

Figure 12. PCR results of 4 virulence gene analysis (**a** = *mgc2*, **b** = *pvpA*, **c** = *gapA*, **d** = *Lp*) showed the specific bands of each gene with different sizes (*mgc2* = 824 bp, *pvpA* = 702 bp, *gapA* = 332 bp, *Lp* = 590 bp).

* means the MG isolates using in pathogenicity study (Experiments 2 and 3)

** means the isolate that is not MG

When compared among 5 isolates by using the pathogenicity tests (experiments 2 and 3), the 58/46, S6 and F strain performed all PCR positive of 4 virulence genes (Table 6). The 31/46 and 54/46 strains showed negative results of *pvpA* gene (Table 6).

Table 6. PCR results of 4 virulence gene analysis

Gene	31/46	54/46	58/46	S6	F
mgc2	+	+	+	+	+
pvpA	-	-	+	+	+
gapA	+	+	+	+	+
Lp	+	+	+	+	+

2. Pathogenicity study in experimentally challenged chickens:

Results showed that the 58/46 isolate induced the most severe lesion scores (Table 7). There were 2 chickens inoculated with 58/46 isolate and only one chicken inoculated with F strain showed mild clinical signs including open mouth breathing and slightly swollen of eyelids (Figures 13, 14). However, those sick birds still had normal feed and water consumption. None of chickens was severe sick or dead during the experiment.

The AS of sick birds were 1 and 2 in the 58/46 challenged birds (Figures 15, 16) and 1 in F strain challenged bird (Figure 17). The TS of sick birds were as same as AS (Figure 19a-c). The tracheal histopathology finding of the sick chickens showed slightly to moderate thickening of the tracheal wall due to the cell infiltration. The cilia had not the defect but epithelial cells were flatted and irregular arranged (Figure 19a-c). The most of euthanized chickens showed normal AS (Figure 18) and TS (Figure 19d), but slight lesion scores were found in some birds.

The mean airsac lesion scores showed that 58/46 isolate had the highest score (1.62) and then followed by F (1.55), S6 (1.45), 31/46 (1.31), 54/46 (1.1) and the control group (0.38). However, statistical analysis revealed significant difference only between 58/46, 54/46 and control group ($p<0.05$) (Table 7). The mean tracheal lesion scores were not significant difference among experimental groups including the control group ($p>0.05$) (Table 7).

ELISA titers of chickens at 21 days old were zero in all samples. At 42 days old or 21 days post inoculation, the ELISA titers still were zero in all birds of all experimental group.

Table 7. Airsac and tracheal lesion scores ($\bar{x}$±S.D.) and %MG PCR positive results in experimental chickens

	Airsac (n= 21)	**Trachea** (n= 7)	**PCR** (n= 7)
21 days old	0.04±0.23	0.28±0.55	0 (0/7)
42 days old			
C	0.38±0.44 [a]	0.64±0.83	0 (0/7)
S6	1.45±0.82 [b,c]	0.71±0.85	71.43 (4/7)
31/46	1.31±0.74 [b,c]	0.89±0.83	100 (7/7)
54/46	1.1±0.77 [b]	0.82±0.83	85.71 (6/7)
58/46	1.62±0.80 [c]	1.11±1.06	100 (7/7)
F	1.55±0.85 [b,c]	0.71±0.91	85.71 (6/7)

[a, b, c] the different superscripts in the same column mean significant difference (p<0.05)

Figure 13. Clinical signs of the second sick chicken challenged with 58/46 isolate: The sick chicken showed mild clinical signs, open mouth breathing and slightly swollen of eyelids.

Figure 14. Clinical signs of the chicken challenged with F strain: The sick chicken showed slightly swollen of eyelids and watery eyes.

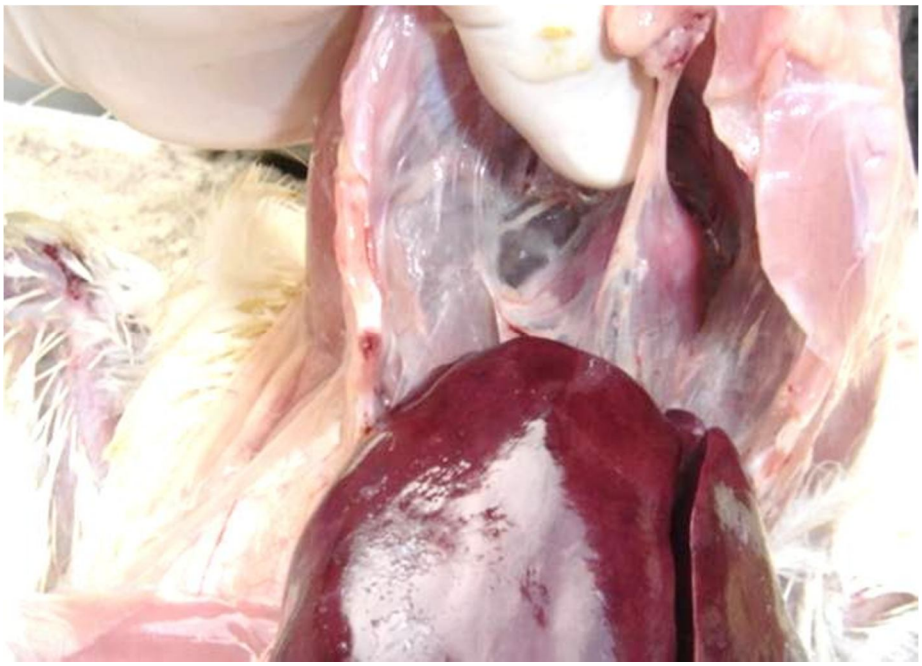


Figure 15. Thoracic airsac of the first sick chicken challenged with 58/46 isolate: Necropsy findings showed slightly thick and presents small accumulations of cheesy exudates (airsac score = 2).

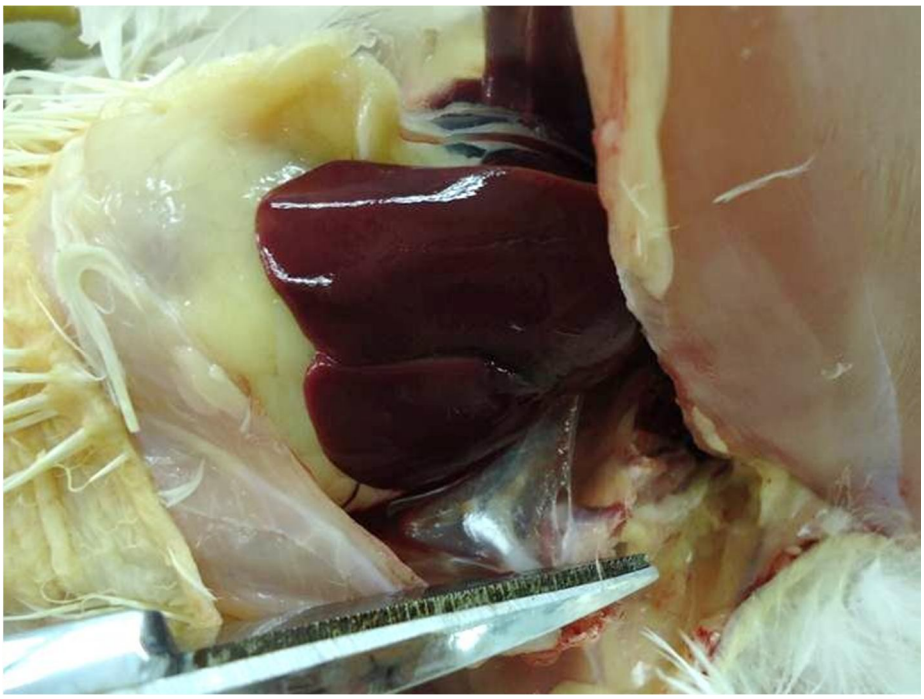


Figure 16. Thoracic airsac of the second sick chicken challenged with 58/46 isolate: Necropsy findings showed slight cloudiness of the airsac membrane (airsac score = 1).

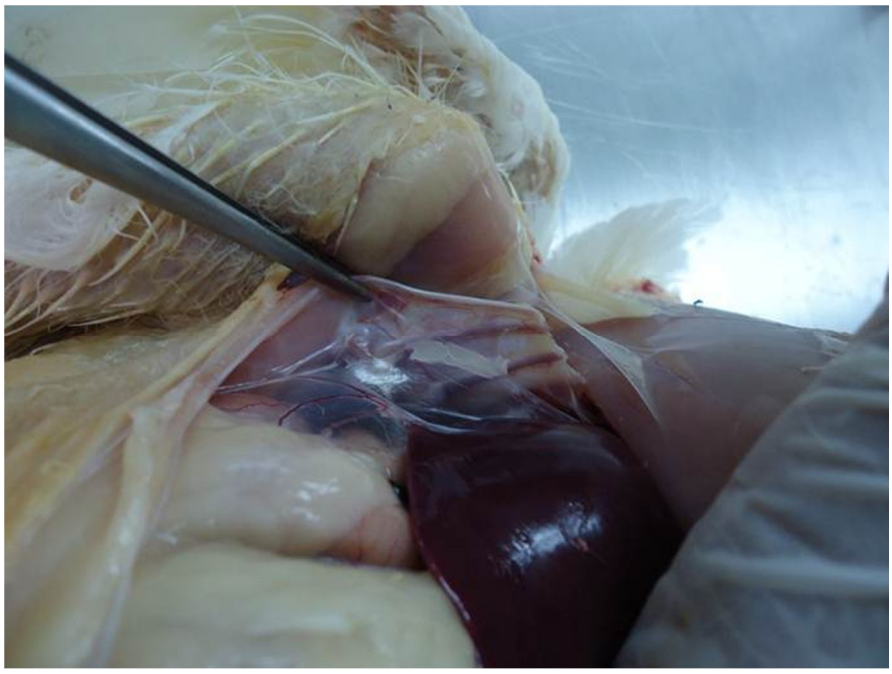


Figure 17. Thoracic airsac of the sick chicken challenged with F strain: Necropsy findings showed slight cloudiness of the airsac membrane (airsac score = 1).

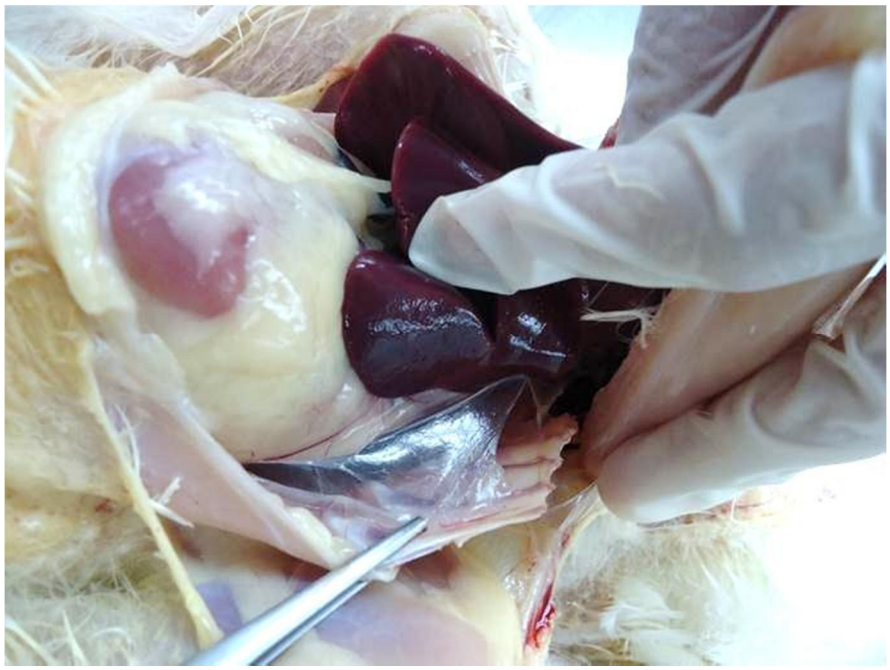
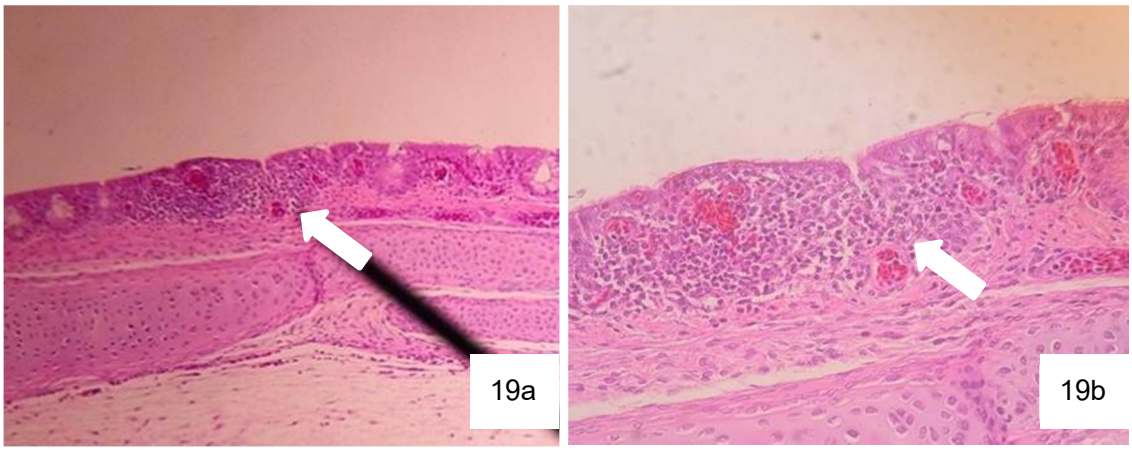


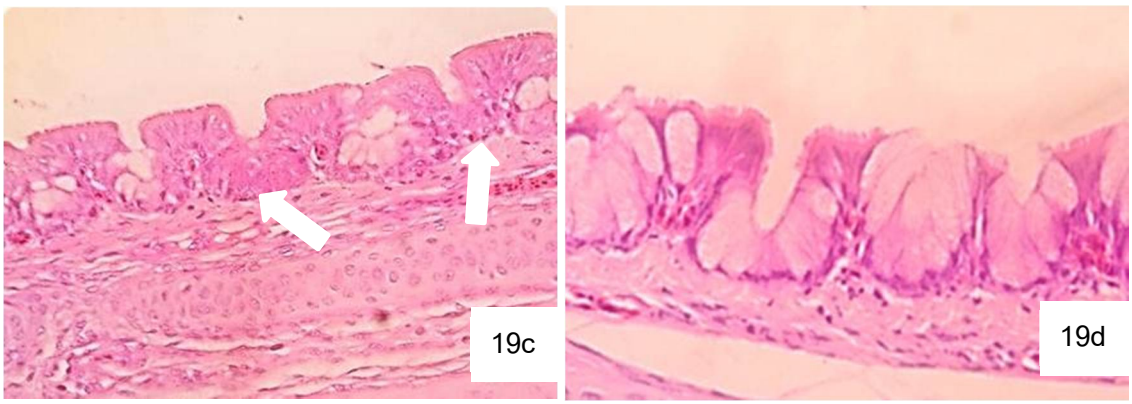
Figure 18. Thoracic airsac of the first euthanized chicken challenged with 31/46 isolate: No thoracic airsac lesion was observed (airsac score = 0).

Figure 19a-d. Histopathological tracheal lesions in chicken infection model.



19a and 19b - 19a (H&E, 40x); 19b (H&E, 100x)

The tracheal histopathology of the first sick chicken challenged with 58/46 isolate showed moderate thickening of the tracheal wall due to the cell infiltration (Tracheal score = 2).



19c – (H&E, 100x)

The tracheal histopathology of the second sick chicken challenged with 58/46 isolate showed slight thickening of the tracheal wall with the small aggregation of cells (Tracheal score = 1).

19d – (H&E, 400x)

No tracheal lesion of the first euthanized chicken challenged with 31/46 isolate was observed (Tracheal score = 0).

3. **Pathogenicity study in chicken embryonated eggs:**

Results showed that the 58/46 isolate induced the most severe lesion scores (Table 8). None of embryos died during incubation. After hatching, 4 chickens of 58/46 isolate were sick and 1 chicken died at day 4 (Figure 20a) with the high lesion scores (AS = 3 and TS = 3) (Figures 21, 22 a-b). Two and one chickens showed mild clinical signs and lesions (Figure 20b), challenged with 31/46 isolate and F strain, respectively. The sick birds challenged with 31/46 isolate showed moderate lesion scores (AS = 2, TS = 2) (Figure 22c-d), whereas a sick bird challenged with F strain had the mild lesion scores (AS = 2, TS = 2) (Figure 22e-f).

The mean airsac lesion scores showed that 58/46 isolate had the highest score (1.04) and followed by F (0.83), 31/46 (0.5), S6 (0.31), 54/46 (0.27) and the control group (0.11). However, statistical analysis revealed significant difference only between the 58/46 isolate and control group ($p<0.05$), but not among experimental groups ($p>0.05$) (Table 8). There were the statistical significance of the mean tracheal scores between 58/46 isolate and S6 strain including control group ($p<0.05$) (Table 8).

Table 8. Airsac and tracheal lesion scores ($\bar{x}\pm$S.D.) and %MG PCR positive results in 8 days old embryos.

	Airsac (n=15)	**Trachea** (n=5)	**PCR** (n=5)
Control	0.11 ± 0.26^{a}	0.1 ± 0.31^{a}	0 (0/5)
S6	$0.31\pm0.47^{a,b}$	$0.35\pm0.59^{a,b}$	80 (4/5)
31/46	$0.5\pm0.64^{a,b}$	$1.05\pm0.94^{b,c}$	100 (5/5)
54/46	$0.27\pm0.39^{a,b}$	$0.7\pm0.86^{a,b,c}$	80 (4/5)
58/46	1.04 ± 1.07^{b}	1.35 ± 0.93^{c}	100 (5/5)
F	0.83 ± 0.87^{b}	$0.9\pm0.97^{a,b,c}$	100 (5/5)

$^{a, b, c}$ the different superscripts in the same column mean significant difference ($p<0.05$)

Figure 20a-b. Clinical signs of experimental chickens in chicken embryonated egg infection model

20a - The 4 days old chicken of 58/46 isolate died after showing severe depression, anorexia and difficult breathing.

20b - chickens challenged with 31/46 and F strain showed mild clinical signs, depression and decrease in water and feed consumption.

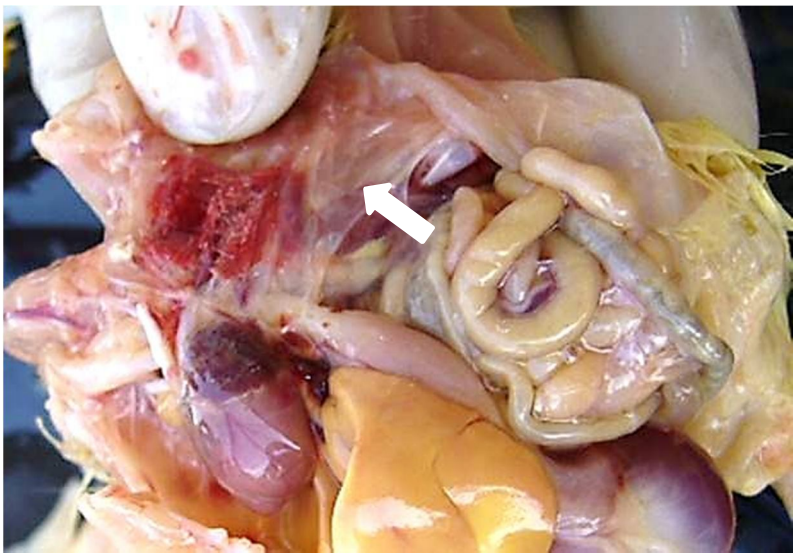
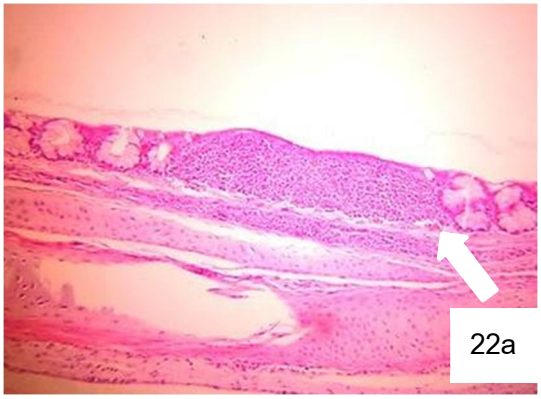
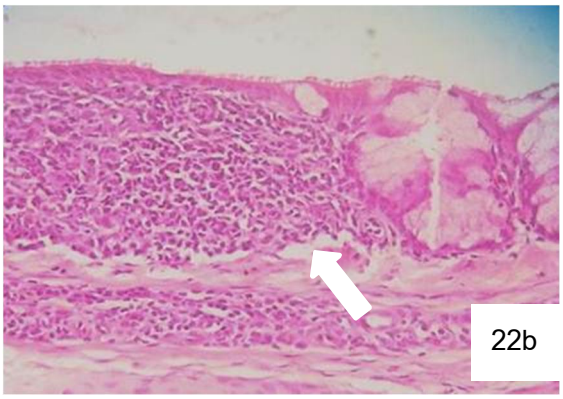


Figure 21. Thoracic airsac of the dead chicken challenged with 58/46 isolate: Necropsy findings showed obviously thick and meaty in consistency, with cheesy exudates in left airsac (airsac score = 3).

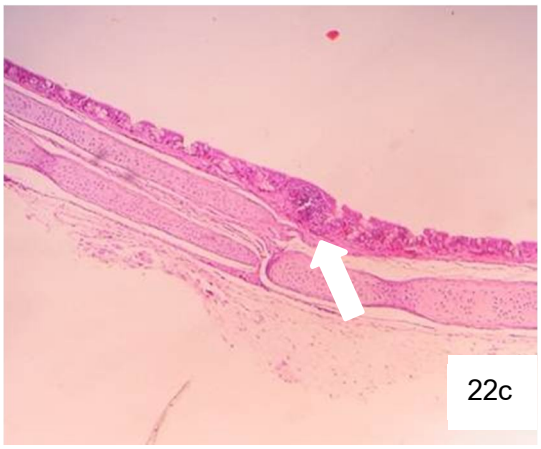
Figure 22a-f. Histopathological tracheal lesions in chicken embryonated egg infection model.

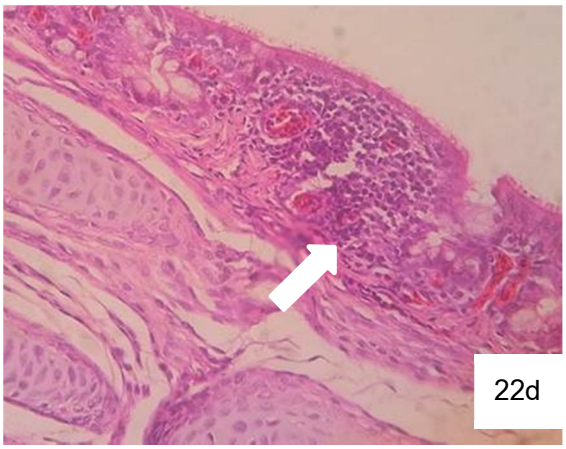




22a and 22b - 22a (H&E, 40x); 22b (H&E, 100x)

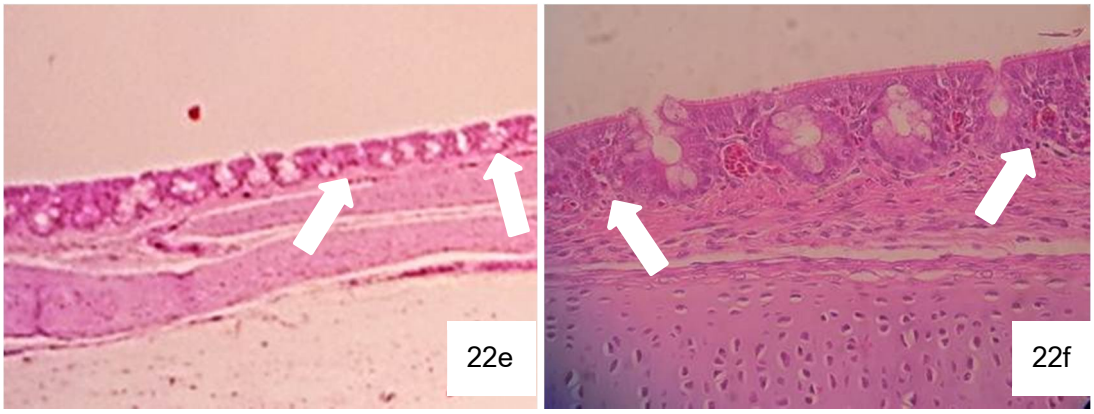
The tracheal histopathology of the dead chicken challenged with 58/46 isolate showed extensive thickening of the wall due to the cell infiltration and edema (Tracheal score = 3).





22c and 22d – 22c (H&E, 40x); 22d (H&E, 100x)

The tracheal histopathology of the sick chicken challenged with 31/46 isolate showed moderate thickening of the tracheal wall due to the cell infiltration (Tracheal score = 2).



22e and 22f – 22e (H&E, 40x); 22f (H&E, 100x)

The tracheal histopathology of the the sick chicken challenged with F strain showed slight thickening of the tracheal wall with the small aggregation of cells (Tracheal score = 1).

CHAPTER V
DISCUSSION

1. Virulence gene analysis:

In present study, 4 genes (*pvpA, gapA, mgc2* and *LP* (*MGA_0319*) gene), which encoding MG surface proteins, were analyzed from 19 field isolates of MG from central, eastern and western parts of Thailand and 2 reference strains including strains F (vaccine strain) and S6 (ATCC® 15302™) by using PCR (Ferguson et al., 2005) (experiment 1) to determine the virulence gene profiles. All of these 4 genes previously reported to be found in the virulent MG strain R_{low} (Papazisi et al., 2003). The results of virulence gene analysis in this study showed that there were 2 genes, *mgc2* and *LP* genes, showed positive PCR results in all of Thai MG isolates including 2 reference strains, whereas some isolates performed PCR negative results of *gapA* and/or *pvpA* genes. These indicated the variation in *gapA* and *pvpA* genes existence in genome among different MG isolates. The *gapA* gene was found in 15 of 19 Thai MG isolates in this work and *pvpA* gene was found in only 4 of 19.

The *gapA* gene encodes GapA protein which is the primary cytadhesin molecule related to cytadhesion process of MG (Goh et al., 1998, Papazisi et al., 2000, Mudahi-Orenstein et al., 2003). This gene have been reported to be found as a single copy in genome of several different strains of MG. There were the intraspecies strain variation in the size of GapA protien from 98-110 kDa. Additionally, the chicken tracheal-ring inhibition-of-attachment assay showed that anti-GapA gene Fab fragments could significantly inhibit MG attachment by 64% (Goh et al., 1998, Mudahi-Orenstein et al., 2003). There were the previous evidence revealed that GapA protien is missing in MG strain R_{high} (passage 164), the laboratory attenuated strain, comparing with strain virulent R_{low} (passage 15) which GapA still remains. Moreover, GapA-negative MG R_{high} performed lower cydadhesin ability and lower in pathogenicity compared with Gap-A positive strain R_{low} (Papazisi et al., 2000).

In this study, 4 of 19 Thai MG isolates performed *gapA*-negative PCR results. Interestingly, all 4 *gapA*-negative isolates also performed *pvpA*-negative PCR results. Additionally, among those 4 *gapA*-negative isolates, 3 isolates were from eastern and 1 isolate was from western part of Thailand, whereas all MG isolates from central part of Thailand showed *gapA*-positive PCR results. This may imply that Thai MG isolates from central part of Thailand have *gapA* expression and perform more virulence than those from other parts. We could confirm this conclusion by pathogenicity test in chicken and chicken embryonated egg (CEE) in experiments 2 and 3. The experiments used Thai MG 58/46 isolate as the representative of MG from the central part of Thailand compared with 31/46, 54/46 isolates from eastern part and 2 reference strains. The results revealed that 58/46 isolate from the central part, which performed *gapA* positive by PCR, caused the most severe clinical sign, lesion scores and mortality. Unfortunately, the pathogenicity between *gapA*-positive and *gapA*-negative isolates was not determined, this point should be further investigated. However, there was the evidence report that GapA cytadhesin plays a role in promoting virulence and host colonization of MG and the GapA deficient isolates can produce the low pathogenicity when compare with the virulent R_{low} (Indiková et al., 2013).

The *pvpA* gene encodes the accessory cytadhesin protein, PvpA, which vary in size among MG strains (Goh et al., 1998; Boguslavsky et al., 2000; Liu et al., 2001; Papazisi et al., 2003). In this study, the *pvpA* gene was found positive PCR results in 4 of 19 Thai MG isolates. This is similar to the previous report by Liu et al. (2001) who found the lower percentage of *pvpA*-positive PCR results compared with MG rRNA PCR test of the same samples. They suggested that the sensitivity of MG *pvpA* PCR test may be less than MG PCR based on rRNA gene. On the contrary, there were the reports revealed that the *pvpA* gene was present in all tested strains with different sizes of PCR products (Boguslavsky et al., 2000; Ferguson et al., 2005). Surprisingly, the most of Thai MG isolates were *pvpA-negative* (15 from 19 isolates). However; the primers used in the present study had the same sequence with previous research which amplified *pvpA* C-terminus-encoding region (Liu et al., 2001; Ferguson et al., 2005).

Beside the accessory role in cytadherence, the size-variant PvpA surface protien also plays the role as epitope of MG recognized by chicken immune system (Yogev et al., 1994; Levisohn et al., 1995; Boguslavsky et al., 2000; Liu et al., 2001). The genetic variation of *pvpA* gene occurred the proline-rich C-terminal region which are identified as major immunogenic surface antigen. These proline-rich repeat units are responsible for pathogen-host cell interaction and to be important in pathogenicity of MG (Yogev et al., 1994; Levisohn et al., 1995; Boguslavsky et al., 2000). In the present study, the immune response of chicken between *pvpA*-positive and *pvpA*-negative isolates was not investigated, only pathogenicity in chicken and CEE were tested.

The results of pathogenicity tests compared between *pvpA*-positive and *pvpA*-negative isolates in chickens and CEE showed that the *pvpA*-positive Thai MG isolate (58/46) produced more virulence than those *pvpA*-negative isolates (31/46 and 54/46). The reference strains, F and S6, also showed *pvpA*-positive results and produced moderate virulence compared with 3 field isolates in chickens and CEE models (Tables 7 and 8). This suggests that *pvpA* gene may involve in virulence and lacking of *pvpA* gene trends to diminish the pathogenicity of MG.

2. Pathogenicity study in experimentally challenged chickens

Pathogenicity test in chicken infection model was performed in 21 days old chickens. The chickens were divided into 6 groups and intranasally inoculated with 3 field isolates (31/46, 54/46 and 58/46), 2 reference strains (F and S6) or Frey's broth (sham-inoculated group), respectively. Results showed that there were 2 chickens inoculated with 58/46 isolate and only one chicken inoculated with F strain showed mild clinical signs. However, those sick birds still had normal feed and water consumption. None of chickens was severe sick or dead during the experiment but the slight macroscopic airsac and microscopic tracheal lesions were found in some birds.

The 58/46 isolate which obtained from the central part of Thailand induced the most severe lesion scores compared with other field isolates from eastern part and reference strains (Table 7). The mean airsac lesion scores showed that 58/46 isoate had the highest score (1.62) and followed by F (1.55), S6 (1.45), 31/46 (1.31), 54/46 (1.1) and the

control group (0.38). However, statistical analysis revealed that the significant difference was observed only between 58/46, 54/46 and control group ($p<0.05$) (Table 7). Interestingly, 58/46, F and S6 which had positive results for all 4 virulence genes (*pvpA, gapA, mgc2* and *LP*-positive strains) produced higher airsac scores than 31/46 and 54/46 isolates which were *pvpA*-negative MG. This indicated that *pvpA* gene may involve in virulent of MG. There was no statistical significance of the mean tracheal scores among experimental groups ($p>0.05$).

Sprygin et al. (2011) studied on pathogenicity of MG field isolates in Russia by intranasal inoculation into 2 weeks old chickens. This study showed that none of the Russian MG field isolates was as virulent as S6 strain. Comparing with F strain, S6 is the more virulent strain which can cause the severe clinical sign and lesions (Levisohn et al., 1986). In contrast, in the present study, the S6 strain was less pathogenic and had lower virulent than F strain. This may involve in the effect of high *in vitro* passage during maintaining and repropagating the organism which was able to reduce in pathogenicity by time (Levisohn et al., 1986). However; in layers, S6 had no significant effect on production of the hens (Basenko et al.,2005; Peebles et al., 2006), whereas the F strain caused delaying and reducing in egg production and egg characteristics alteration (Burnham et al., 2002[a, b]).

The tracheal histopathology finding of the sick chickens showed slightly to moderate thickening of the tracheal wall due to the cell infiltration. The cilia did not have the defect but epithelial cells were flatted and irregular arranged. These suggested that tracheal epithelium may be affected by infection and were in the recover stage at the time of sample collection (at terminated day). Therefore, additional sample collection during experimental period may be needed for following up the histopathologic change in tissue.

ELISA titers of chickens at 21 days old were zero in all samples. At 42 days old or 21 days post inoculation, the ELISA titers still were zero in all birds of all experimental group. ELISA detected IgG which there is about more than 10 days interval that the test may not work properly for diagnosis in early infection stage (Kleven, 1998). It is possible that

ELISA test used in this study may not detect the antibodies titer at 21 days post inoculation. This suggested that the time interval after inoculation should be prolonged for ELISA test.

3. Pathogenicity study in chicken embryonated eggs:

Pakpinyo et al. (2011) reported that the use of CEE to evaluate the virulence or pathogenicity of MG field isolates instead of chickens is possible. After the experiment 2 performed in the chicken infection model, the pathogenicity of 3 MG field isolates and 2 reference strains were also tested in CEE inoculated via yolk sac route. Results showed that none of embryo died during incubation. After hatching, the chickens were subsequently raised to observed the clinical signs and mortality and then euthanized and evaluated for AS and TS. There were 4 chickens of 58/46 isolate were sick and 1 chicken died at day 4 with the high lesion scores. Two and 1 chickens challenged with 31/46 and F strain, respectively showed mild clinical signs and lesions. Results of AS and TS of 7 days old chickens revealed that the 58/46 isolate, collected from the central part, induced the most severe lesion scores (Table 8). There was the significant difference between the 58/46 isolate and control group ($p<0.05$), but not among experimental groups for AS ($p>0.05$). There were the statistical significance between 58/46 isolate and S6 strain including control group for TS ($p<0.05$). This can be concluded that the 58/46 field isolate from the central part of Thailand trends to have the highest pathogenicity compared with other field isolates from the eastern part and reference strains both in chicken and CEE infection models.

In the past, Levisohn et al. (1985) inoculated MG via yolk sac and calculated LD50. The results showed that the highly pathogenic strains had a relationship between the dose of MG inoculation, embryonic death and time of death. In Thailand, there was the previous study on the pathogenicity of Thai MG in CEE compared with vaccine strains (F and 6/85) (Pakpinyo et al., 2011). Results revealed that there were embryonic death during hatching and then the surviving chickens which were continuously raised for 7 days also died during raising period, and the thoracic airsac lesions from Thai MG

inoculation were found in those surviving chickens (Pakpinyo et al., 2011). We agree that using CEE for pathogenicity evaluation is convenient, requires shorter duration, have uncomplicated raising management of chickens when compared with raising laboratory chickens for chicken infection model. This *in ovo* infection model should be the beneficial tool used to study the pathogenicity of other Thai MG isolates in the future.

Conclusions

1. There were the variation in *gapA* and *pvpA* genes existence in genome among different MG field isolates. Most of Thai MG isolates were *pvpA*-negative (15/19).
2. All 4 Thai MG isolates, which were *gapA*-negative, also performed *pvpA*-negative results.
3. The *pvpA*-negative Thai MG trends to have lower pathogenicity than those which were *pvpA*-positive.
4. All Thai MG isolates which obtained from central part were *gapA*-positive and their representative (58/46 isolate) produced the most severe lesions in pathogenicity tests in chickens and CEE comparing to MG from other parts of Thailand.
5. The reference strains (F and S6) performed positive results for all 4 virulence genes and had moderate pathogenicity in chicken and CEE infection models.

Additional comments

Further studies may be needed to compare the pathogenicity between *gapA*-positive and *gapA*-negative isolates, also between isolates lacking of *pvpA* or *gapA* and isolates lacking of both *pvpA* and *gapA*. Moreover, the research on immunogenic effects when MG lacks of *pvpA* should be additional study to confirm the role of *pvpA* gene on antigenicity.

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APPENDICES

APPENDIX A

Evaluation of lesion scores of gross thoracic airsacs (Kleven et al., 1972)

Lesion scores of thoracic airsacs were determined by visual observation and scored from 0 to 4, as the following (Figure 23);

0: No airsac lesion is observed,

1: Lymphofollicular lesions or slight cloudiness of the airsac membrane are found.,

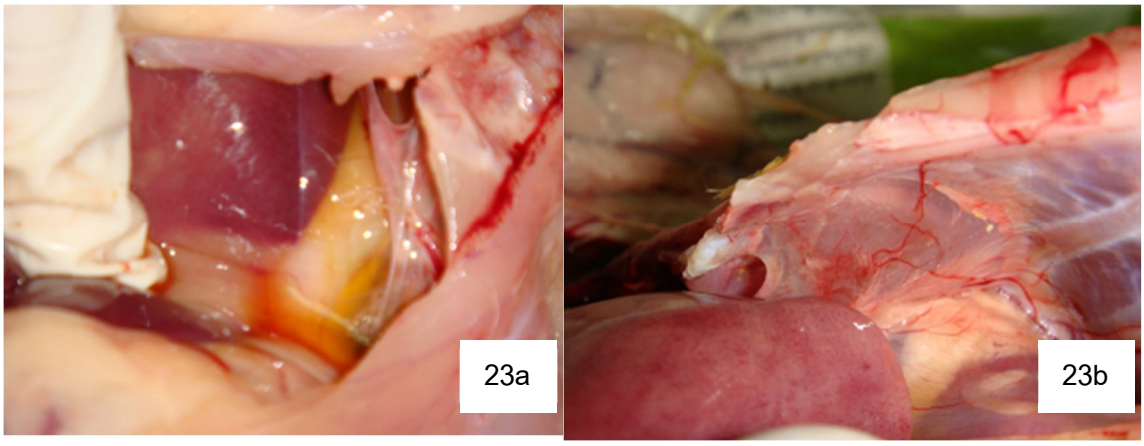
2: Airsac membrane is slightly thick and usually presents small accumulations of cheesy exudates.,

3: Airsac membrane is obviously thick and meaty in consistency, with large accumulations of cheesy exudates in one airsac.,

4: Lesions are observed as same as 3, but 2 or more airsacs are found.

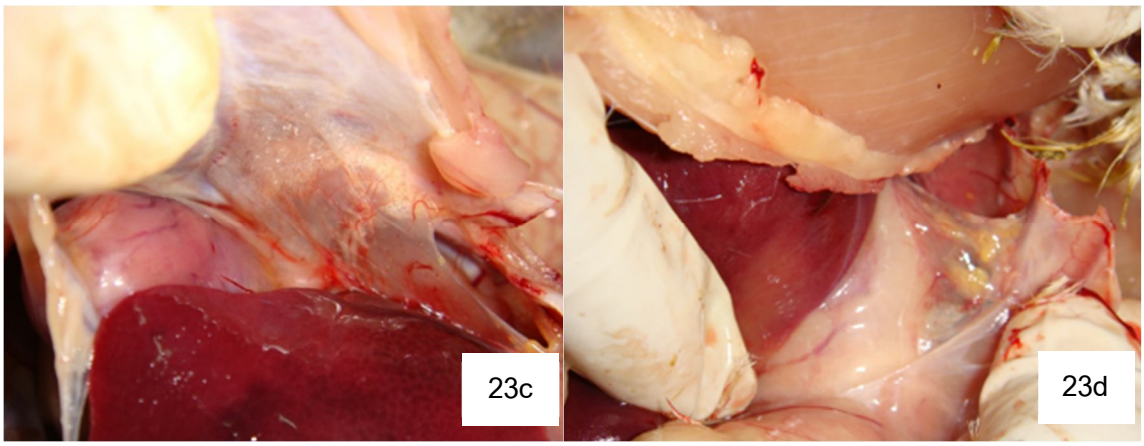
Figure 23a-d - Macroscopic lesion characteristics of each level of airsac score (From 0-4, respectively)

(contributed by Assoc.Dr.Somsak Pakpinyo, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University)



23a – Macroscopic lesion characteristics of airsac score 0

23b – Macroscopic lesion characteristics of airsac score 1



23c – Macroscopic lesion characteristics of airsac score 2

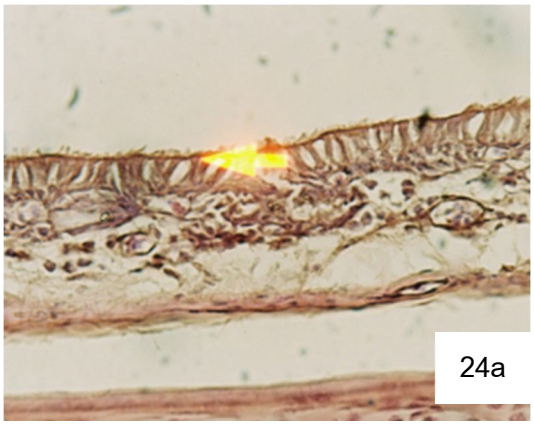
23d – Macroscopic lesion characteristics of airsac score 3

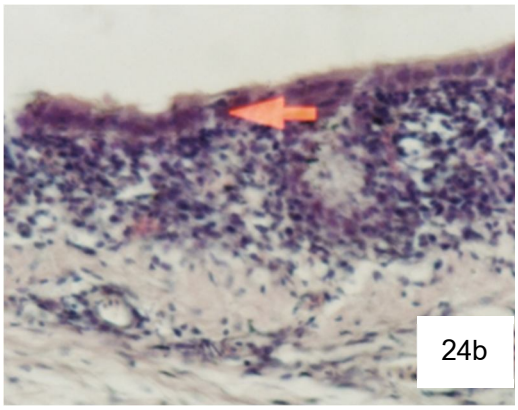
APPENDIX B

Evaluation of lesion scores of histopathologic trachea (Yagihashi and Tajima, 1986)

1. Seven tracheas from each group were collected and fixed in 10% formalin solution for 24 hours for histopathology.
2. During tissue processing, each trachea was crossly sectioned into 4 pieces (1 proximal, 2 middle, 1 distal part of trachea).
3. All tissue slides were stained with hematoxylin and eosin (H&E).
4. Lesion scores of tracheas were blindly investigated under microscopic condition.
5. Histopathological tracheal lesion scores were determined and scored from 0 to 3, as the following (Figure 24):
    - 0: No significant changes are observed.,
    - 1: Small aggregation of cells (mainly lymphocytes) is found.,
    - 2: Moderate thickening of the wall due to the cell infiltration, and edema commonly accompanied with epithelial degeneration and exudation is present.,
    - 3: Extensive thickening of the wall due to the cell infiltration with or without exudation is determined.

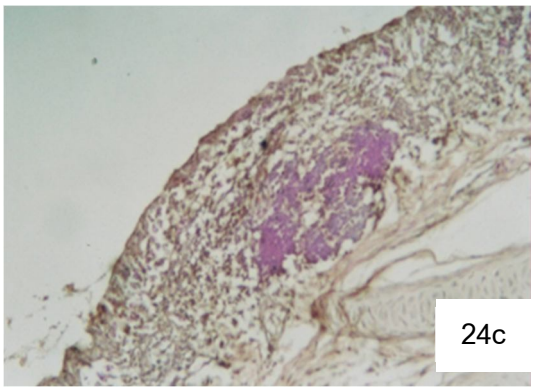
Figure 24a-d - Histopathological tracheal lesion score (score 0 to 3, respectively) (contributed by Assoc.Dr.Somsak Pakpinyo, Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University)

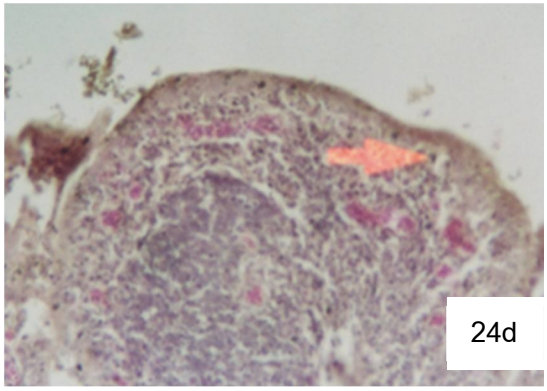




24a – Histopathological tracheal lesion score 0

24b – Histopathological tracheal lesion score 1





24c – Histopathological tracheal lesion score 2

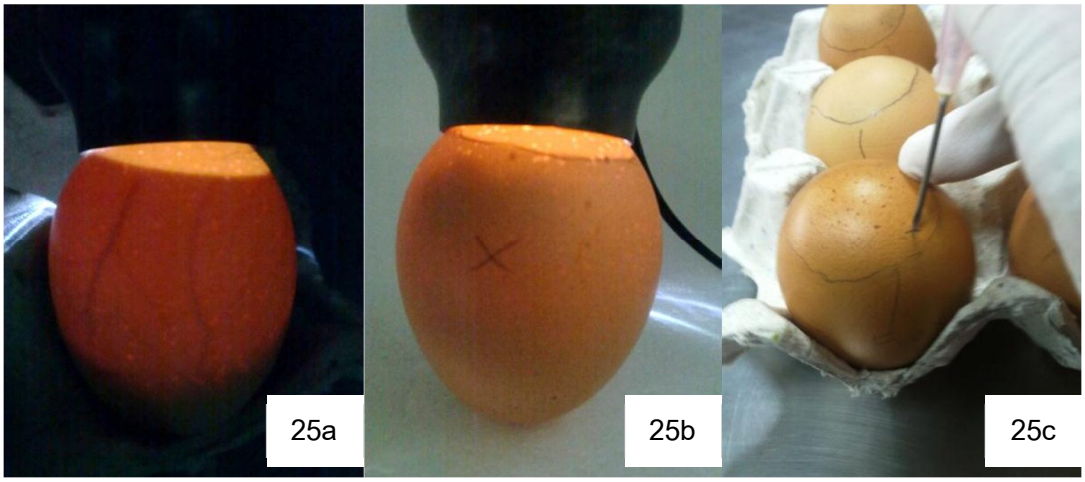
24d – Histopathological tracheal lesion score 3

APPENDIX C

Method of embryonated egg inoculation via yolk sac route (Pelczar et al., 2010)

1. Candle the embryonated egg and mark the air cell border and positions of embryo, blood vessels and yolk sac on egg shell (Figure 25).
2. Puncture through the egg shell with 1 inch gauge no.18 needle to make a small hole over air cell border for MG inoculation.
3. Insert 1 ½ inches gauge no.21 (or smaller size) needle connected with syringe through the hole to deliver the inoculums straight into the yolk sac.
4. Before injection, make sure that the tip of needle is inside the yolk sac by drawing yolk content backward into the syringe, and then inject inocula gently into the yolk sac.
5. Seal a hole on egg shell with the tape or candle wax and subsequently incubate in the egg incubator.

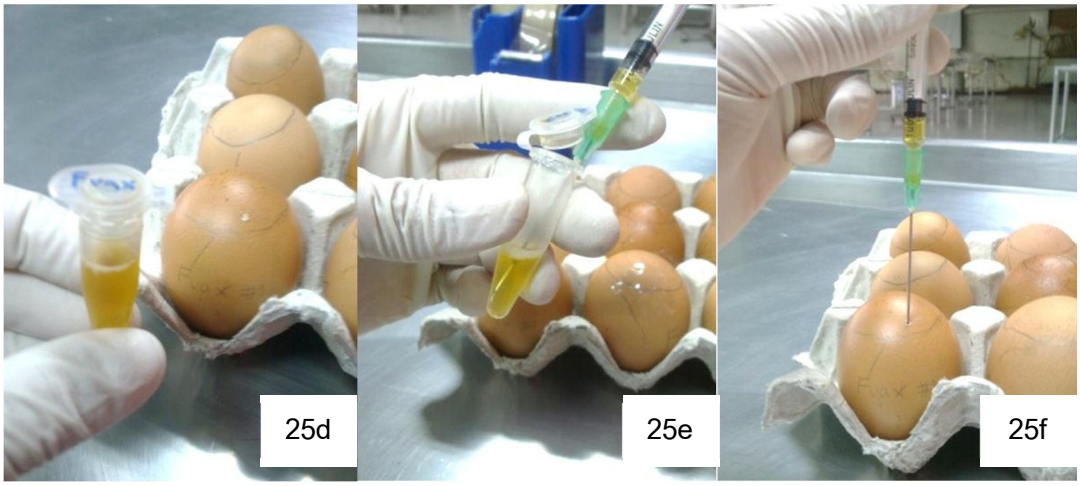
Figure 25a-i. Procedures of MG inoculation into yolk sac of 8-day-old embryonated egg



25a – Candle the 8-day-old embryonated egg

25b – Mark the position of embryo on the egg shell

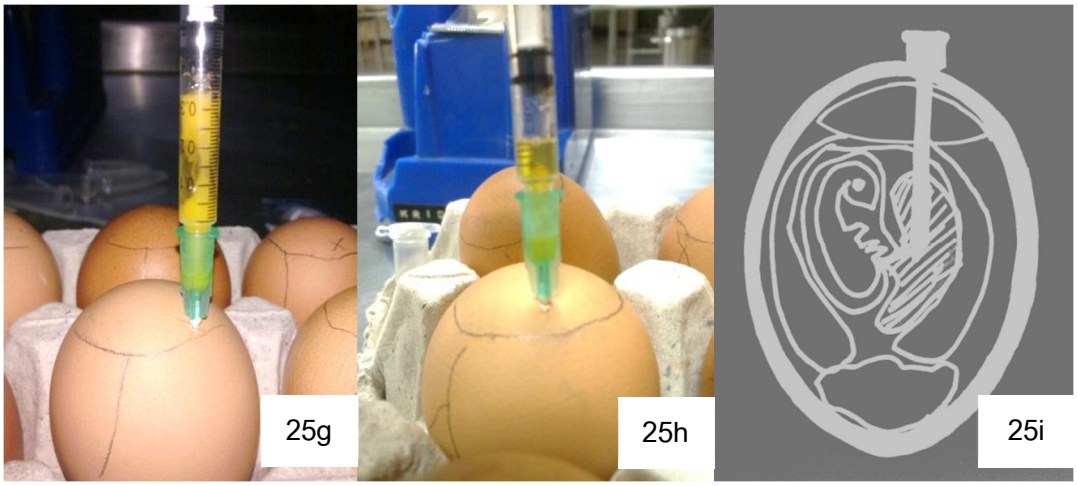
25c – Punch egg shell with 1 inch gauge no.18 needle



25d – Thaw the frozen inocula

25e – Prepare 0.1 ml inocula in syringe connected with 1 ½ inches gauge no.21 (or smaller size) needle

25f – Insert the tip of needle through the hole straight into the yolk sac



25g – Draw the yolk content backward to check the needle tip position

25h – Inject inocula into the yolk sac gently

25i – Position of needle tip inside the egg

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