Original Article

Analysis of Virulence Genes and Pathogenicity of Thai *Mycoplasma gallisepticum* Isolates

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Abstract

Mycoplasma gallisepticum (MG) causes chronic respiratory disease in poultry. MG organisms consist of several virulence genes which are related to infection. The aim of this study was to analyze the virulence genes and determine the pathogenicity of Thai MG isolates. In this study, there were 3 experiments; nineteen MG isolates; and 2 reference strains, F and S6 strains, analyzed to detect 4 virulence genes (*LP*, *gapA*, *pvpA* and *mgc2*) by PCR assay (experiment 1). The pathogenicity study of sham negative control group, S6 strain, 3 selected isolates (AHRL 31/46, 54/46 and 58/46) and F strain, respectively, were inoculated into 6 groups of 21-day-old chickens, 21 birds in each group (experiment 2), and 6 groups of 8-day-old chicken embryonated eggs (CEEs), 15 eggs in each group (experiment 3). In experiment 2, at 42 days old, all birds were bled for MG ELISA, swabbed for MG PCR assay and necropsied to evaluate gross thoracic air sac and microscopic tracheal lesion scores. In experiment 3, after hatching, all chicks of each group were separately raised until 7 days old. At 7 days old, all birds were swabbed and necropsied as previously described. Results revealed that all MG isolates and reference strains had all virulence genes. The pathogenicity study in chickens was similar to that of CEEs. The Thai MG AHRL 58/46 isolate could induce the most severe clinical signs and lesions of air sacs and tracheas compared with those of the other groups. This study revealed that Thai MG isolates could cause disease in chickens.

Keywords: chickens, embryonated chicken eggs, *Mycoplasma gallisepticum*, pathogenicity, Thailand, virulence genes

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Introduction

Mycoplasma gallisepticum (MG) infection is an important respiratory disease of chicken called chronic respiratory disease (CRD) or air sac disease which causes significant economic loss in the poultry industry. Moreover, MG infection is related to carcass condemnation from under-targeted body weight and lesions of air sacs (Evans et al., 2005).

Clinical signs of MG infection in chicken include respiratory rales, coughs, sneezes, nasal discharge and conjunctivitis. The disease can transmit to normal chickens by aerosol and transovarian routes. Other important characteristics are slow development of clinical signs and long course of disease (Ley, 2008). Once an outbreak occurs, the disease will be rapidly spread within the flock and forms chronic disease which is difficult to be controlled by antibiotics. Chronic MG infection occurring within the flock implies that chicken immunity cannot completely eliminate MG organisms (Levisohn et al., 1995; Glew et al., 2000).

In the past, scientists believed that MG was an extracellular organism. However, recently it has been revealed that MG is an intracellular organism which is resistant to host immunity and antibiotics (Winner et al., 2000). After entering the host, MG primarily grows in the respiratory tract, but other organs such as reproductive tract, brain and eyes are also targeted (Ley, 2008). An important mechanism in pathogenesis of MG is cytadherence with host epithelial cell surface before cell proliferation (Ley, 2008). The cytadhesion is related to variable cytadhesin proteins 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 (Boguslavsky et al., 2000). Those molecules are required to work together in the cytadherence process, therefore, lack 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; Boguslavsky et al., 2000; Papazisi et al., 2002).

Numerous researches on MG genetics have been developed after Papazisi et al. (2003) demonstrated whole genome of R_{low} strain. Several genes were used for genetic variation studies such as *16S rRNA*, *mgc2*, *mgc1 (gapA)*, *pvpA* and surface lipoprotein (*LP*) genes (Ferguson 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), in relation to 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. Results showed the genetic variation with insertion and/or deletion of genes and also the epidemiological association with disease outbreak in those areas. An 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 occurred in R_{high} strain resulting in attenuation of the organism (Szczepanek, 2009).

Virulence gene analyses of MG mostly study genes related to surface molecule of cell membrane which play 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. Important proteins of MG which function in host immune response are adhesins or hemagglutinins. These proteins will bind to receptor site on host epithelium, resulting in MG colonization and then progression of infection from this location (Ley, 2008).

In Thailand, a preliminary study of Pakpinyo et al. (2011) of the pathogenicity in CEF of MG isolates from broilers compared with S6 strain found that Thai MG isolate had more pathogenicity than the S6 strain. However, an analysis of Thai MG virulence genes has not been reported. The aims of this study were to analyze 4 virulence genes (*LP* (*MGA*_0319), *gapA*, *pvpA* and *mgc2* gene) of the Thai MG isolates by using PCR assay and to evaluate the pathogenicity of 3 geographically different isolates of the Thai MG isolates compared with two reference strains (F and S6) in experimental chickens and CEE.

Materials and Methods

***M. gallisepticum* isolates:** Nineteen MG isolates were used and obtained from broiler breeder, broiler and layer farms in the central, western and eastern parts of Thailand during 2003-2004. All isolates obtained from the farms were isolated and cultured in Frey's broth medium supplemented with 15% swine serum (FMS) (Kleven, 1998) at 37°C under aerobic condition until the broth color changed from red to orange yellow. The cultured broth was plated on FMS agar at 37°C under aerobic condition. MG colonies were identified by direct immunofluorescent assay (Kleven, 1998) using fluorescein-conjugated rabbit antiserum provided by S.H. Kleven (Department of Avian Medicine, University of Georgia, Athens, GA). All MG isolates were propagated in FMS at 37°C under aerobic condition until log phase and stored at -80°C. All frozen isolates were re-propagated in FMS at 37°C under aerobic condition until log phase before used in experiments. Two reference strains including MG S6 strain (ATCC® 15302™) and MGF strain (F-Vax MG® Intervet Schering-Plough Animal Health, Thailand) used in this study were propagated in FMS as previously described.

Experimental designs

Experiment 1: virulence gene analysis. Four virulence genes (*LP* (*MGA*_0319), *gapA*, *pvpA* and *mgc2* gene) were analyzed in this experiment by PCR assay. Primers of the 4 virulence genes were described by Ferguson et al. (2005) (Table 1). Amplification conditions for all 4 genes were 95°C for 5 min, and 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 45 s, and 72°C for 5 min. Each reaction was performed concurrently with distilled water as a negative control.

Expected amplification products of *LP*, *gapA*, *pvpA* and *mgc2* genes were 590, 332, 702 and 824 bp, respectively.

Experiment 2: chicken experiment. One hundred and fifty six, one-day-old commercial broiler chickens obtained from MG free broiler breeders were raised in wired cages in separated rooms until 21 days old. At 21 days old, 30 chickens were randomly sampled for MG status before starting the experiment. For MG status, blood collection was determined by MG ELISA test kits. Then, the birds were euthanized to necropsy for evaluation of gross thoracic air sac lesion scores. Seven tracheas were evaluated for histopathological tracheal lesion scores. Each trachea was crossly sectioned into 4 pieces (1 proximal, 2 middle, 1 distal part of trachea). The thoracic air sacs of all individual necropsied birds were swabbed and then the swabs were pooled as 10 samples to detect MG DNA by PCR assay. The remaining chickens were divided into 6 groups of 21 birds each. All birds were inoculated with 0.1 ml of either the FMS broth medium or MG cultured broth containing approximately $1.0x10^6$ CFU via intranasal route as follows. Groups 1-6 served as sham negative control, MG S6, 3 Thai MG isolates: AHRL 31/46, 54/46 and 58/46, and MGF strain, respectively. Clinical signs and mortality were observed daily until 42 days old. Dead birds were necropsied and their thoracic air sacs and tracheas were blindly evaluated as previously described. At 42 days old, all remaining birds were bled for MG ELISA titers, euthanized, and necropsied to evaluate the thoracic air sacs as previously described. Then, tracheal swabs were done to detect MG DNA by PCR assay. Seven tracheas of each group were submitted for histopathology as previously described. Lesion scores of the air sacs and tracheas were blindly investigated.

Table 1 Sequence of primers (Ferguson *et al.*, 2005) used in virulence gene analysis

Primers	Sequence (5′→ 3′)
lp 1F	CCAGGCATTTAAAAATCCCAAAGACC
lp 1R	GGATCCCATCTCGACCACGAGAAAA
gapA 3F	TTCTAGCGCTTTAGCCCTAAACCC
gapA 4R	CTTGTGGAACAGCAACGTATTCGC
pvpA 3F	GCCAMTCCAACTCAACAAGCTGA
pvpA 4R	GGACGTSGTCCTGGCTGGTTAGC
mgc2 1F	GCTTTGTGTTCTCGGGTGCTA
mgc2 1R	CGGTGGAAAACCAGCTCTTG

Experiment 3: chicken embryonated eggs (CEEs) experiment. Ninety, 8-day-old CEEs were mycoplasma free and were obtained from the experimental layer farm of Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand. The CEEs were divided into 6 groups, 15 eggs each. All CEEs were inoculated with 0.1 ml of either the FMS broth medium or MG cultured broth containing approximately $1.0x10^6$ CFU into yolk sac. All groups were designed as same as experiment 2. The CEEs were incubated in an incubator. The inoculated embryonic eggs were candled twice daily. After hatching, all chicks of each group were separately raised until 7 days old. At 7 days old, all chickens were euthanized, necropsied and evaluated as same as in experiment 2. For MG detection, thoracic air sacs of each necropsied bird were swabbed and then 3 swabs were pooled as 1 sample to be further determined by MG PCR assay.

Air sac lesion score. Air sac lesion score was grossly evaluated as the following criteria: 0 = no air sac lesion was observed, 1 = air sac membrane was slightly thick and usually presented small accumulations of cheesy exudates, 2 = air sac membrane was obviously thick and meaty in consistency, with large accumulations of cheesy exudates in one airsac, and 4 = lesions were observed as same as 3, but 2 or more air sacs were found (Kleven et al., 1972).

Tracheal lesion score. Tracheal lesion score was microscopically evaluated as the following criteria: 0 = no significant lesions were observed, 1 = small aggregate of cells (mainly lymphocytes) was found, 2 = moderate thickening of the wall due to cell infiltration, and edema commonly accompanied with epithelial degeneration and exudation was present, and 3 = extensive thickening of the wall due to cell infiltration with or without exudation was determined (Yagihashi and Tajima, 1986).

Enzyme linked immunosorbent assay (ELISA). Sera were tested with commercial test kits (Synbiotics®, San Diego, CA) following the manufacturer's directions. Briefly, diluted sera were added onto an MG antigen-coated plate, incubated, washed, and peroxidase labeled. Then, an anti-chicken antibody (conjugated antibody) was added. After incubation, the plate was again washed before a substrate and a stop solution were added. The plate was read in an ELISA reader (Labsystems Multiskan MS Type 352, Finland). Optical density of the negative and positive controls and samples were calculated and interpreted according to the manufacturer's recommendations. Titer levels of 0-148, 149-743, and equal or higher than 744 were negative, suspicious, or positive reactors, respectively.

MG PCR assay. The preparation of MG DNA was determined using previously described procedure (Lauerman, 1998). Briefly, one ml of FMS broth sample was pelleted by centrifugation at 13,000 xg, washed twice with distilled water, followed by dilution of the pellet with distilled water, boiling for 10 min, then placing at -20°C for 10 min. The procedure ended with centrifugation and collection of supernatant at 4°C for further PCR. PCR mixture of 50 µl volume consisted of KCl 500 mM, Tris-HCl (pH 8.3) 100 mM, dNTP (Fermentas) 1 mM, primer F (5′GAGCTAATCTGTAAAGTTGGTC3′) and primer R (5′GCTTCCTTGCGGTTAGCAAC3′) (Qiagen) 10 pmole each, Taq polymerase (Fermentas) 1.25 U and DNA template 5 µl (250 ng). MG strain S6 (ATCC 15302) was used as a positive control. PCR mixtures were amplified in a DNA thermal cycler (PCR Sprint,

Thermo Electron Corporation, Milford, MA) at 94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec for 40 cycles followed by 72°C for 5 min. PCR product was analyzed in 2% agarose gel (Pharmacia Biotech AB, Uppsala, Sweden), stained with ethidium bromide, visualized by UV transilluminator, and photographed.

Statistical analysis. Number of sick and dead chickens were analyzed by using Chi square test. Lesion scores of gross thoracic air sacs and histopathological trachea were analyzed by using Kruskal-Wallis test and Mann-Whitney U test at 95% confidence level ($p < 0.05$).

Results

All Thai MG isolates had all virulence genes (*LP*, *gapA*, *pvpA* and *mgc2*) detected by the PCR assay.

In experiment 2, at 21 days old, all 30 birds were negative for the MG ELISA titers. The gross thoracic air sac and histopathological tracheal lesion scores of the necropsied birds were 0.04 ± 0.23 (n = 30) and 0.28 ± 0.55 (n = 7), respectively. The PCR assay did not detect MG DNA of 10 tested samples (data not shown). Mild clinical signs including open mouth breathing and slight swelling of eyelids were observed in groups 5 (AHRL 58/46) and 6 (MGF strain); however, these birds still had normal feed and water consumption. For the birds presenting the mild clinical signs, the tracheal histopathology findings showed slight to moderate thickness of the tracheal epithelial without loss of cilia (Fig 1). At 42 days old, the MG ELISA titers were negative in all samples. For the evaluation of the thoracic air sacs and tracheas, group 5 showed the most severe lesion scores; however, no significant difference in the tracheal lesion scores was observed in all groups. MG PCR was detected in all MG-inoculated groups (Table 2). None of the chicken was sick or died during the experiment.

In experiment 3, no embryonic death was observed during the incubation. After hatching, 4 birds of group 5 were sick and one of them died at day 4 after showing severe depression, anorexia and difficult breathing. The highest lesion scores of the thoracic air sacs were found. Two birds of group 3 (AHRL 31/46) and one bird of group 6 showed mild clinical signs and depression and decrease in water and feed consumption. At 7 days old, group 5 had the most severe lesion scores of thoracic air sacs and tracheas (Table 3).

Table 2 Gross thoracic air sac and histopathological tracheal lesion scores (mean ± SD) and % MG positive by PCR assay of experimental chickens at 42 days old

Group	Thoracic air sacs (n=21)	Tracheas (n=7)	% MG positive by PCR assay (n=7)
1	0.38±0.44 [a]	0.64±0.83	0
2	1.45±0.82 [b,c]	0.71±0.85	71.43
3	1.31±0.74 [b,c]	0.89±0.83	100
4	1.10±0.77 [b]	0.82±0.83	85.71
5	1.62±0.80 [c]	1.11±1.06	100
6	1.55±0.85 [b,c]	0.71±0.91	85.71

[a, b, c]: different superscripts in the same column mean statistical difference ($p<0.05$).

Table 3 Gross thoracic air sac and histopathological tracheal lesion scores (mean ± SD) and % MG positive by PCR assay of experimental chicken embryonated eggs at 7 days old

Group	Thoracic air sacs (n=15)	Tracheas (n=15)	% MG positive by PCR assay (n=5)
1	0.11±0.26 [a]	0.10±0.31 [a]	0
2	0.31±0.47 [a,b]	0.35±0.59 [a,b]	80
3	0.50±0.64 [a,b]	1.05±0.94 [b,c]	100
4	0.27±0.39 [a,b]	0.70±0.86 [a,b,c]	80
5	1.04±1.07 [b]	1.35±0.93 [c]	100
6	0.83±0.87 [b]	0.90±0.97 [a,b,c]	100

[a, b, c]: different superscripts in the same column mean statistical difference ($p<0.05$).

Discussion

This study determined the 4 virulence genes and pathogenicity of Thai MG isolates. The four virulence genes (*LP* (*MGA*_0319), *gapA*, *pvpA* and *mgc2* gene) encoding the MG surface proteins and/or variable cytadhesin proteins were detected in all Thai isolates. The lack of some variable cytadhesin proteins results in loss of infectivity (Razin and Jacob, 1992). The virulent MG strain R_{low}, which is known as pathogenic for chickens, also expressed these virulence genes (Levisohn et al., 1986; Papazisi et al., 2003). However, several consecutive passages cause the loss of virulence genes, for example, the *gapA* gene encoding GapA cytadhesin protein of MG strain R_{high} (passage 164) comparing with MG strain R_{high} (passage 15) (Papazisi et al., 2003). Moreover, GapA-negative MG R_{high} performed decreasing cytadhesin ability and lower in pathogenicity compared with Gap-A positive strain R_{low} (Papazisi et al., 2000). Unfortunately, the present

study did not determine other virulence genes including *crmA* and *vlhA* genes (Papazisi et al., 2002; Papazisi et al., 2003). These genes should be determined in further studies.

The results of pathogenicity tests comparing between different MG isolates in chickens and CEEs showed that group 5 (AHRL 58/46) was more virulent than the other groups including the reference and vaccine strains. This isolate was the virulent strain in Thailand (Pakpinyo et al., 2011). The virulence of the S6 strain is the contrary. The S6 strain is considered as a virulent strain in the field and more virulent than the MGF strain (Levisohn et al., 1986), but it is not in the present study and other publications (Parker et al., 2002; Parker et al., 2003). In addition, the S6 strain did not cause any significant effects on egg production in layer chickens (Basenko et al., 2005; Peebles et al., 2006), whereas the F strain caused delay and reduction in egg production and alteration of egg characteristics (Burnham et al., 2002a,b). A possible reason is the number of passages; the high *in vitro* passages during the maintaining and repropagating MG organisms was able to reduce in pathogenicity by time (Levisohn et al., 1986).

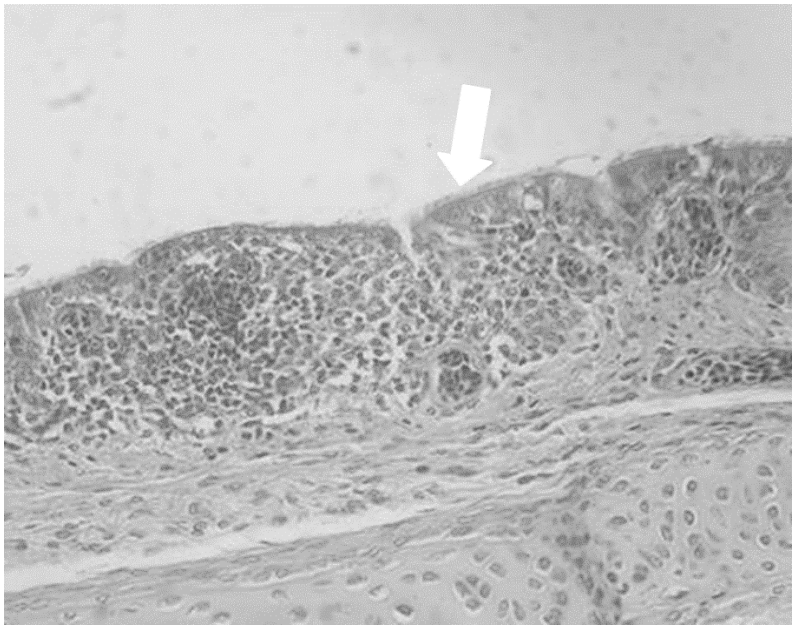

Figure 1 The tracheal histopathology of the sick chicken challenged with 58/46 isolate showed moderate thickening of the tracheal epithelial due to cellular infiltration and edema (Tracheal score = 2; H&E, 100x). The arrow shows normal cilia with flattened and irregularly arranged epithelial cells.

The present study did not find any significant differences in the tracheal lesion scores, whereas the morphology of tracheal epitheliums was flattened and had irregular arrangement, suggesting that the tracheal epitheliums were in recovery stage. A possible reason was that the tracheal collection 3 weeks post inoculation possibly resulted in epithelial regeneration. Several publications described that embryos or chickens inoculated with MG organisms showed tracheal epithelium lesions including losses of cilia, edema or infiltration of inflammatory cells after 6 h of inoculation (Dykstra et al., 1985) or 14 d of inoculation (Lam, 2003). However, the tracheal epithelium of the sick chickens showed slight to moderate thickness caused by the cellular infiltration and edema. Therefore, additional sample collections during experimental period may be needed for following up the histopathologic change in tissue.

The MG ELISA titers of the chickens at 21 and 42 days old were negative in all samples. At 21 days old, the test kits could not detect MG titer in all 30 birds, suggesting that the MG maternally derived antibody of the birds disappeared or all 30 birds were negative against the MG antibody. Unfortunately, the present study did not find positive results by using the ELISA test kits in MG inoculated groups at 42 days old. The ELISA may not work properly for diagnosis in early infection stage (Kleven, 1998). This suggests that the time interval after inoculation should be prolonged for using ELISA test kits or the serum plate agglutination detecting IgM should be used instead of the ELISA test kits.

In conclusion, the present study further evaluated the virulence or pathogenicity of MG isolates in CEEs as described by Pakpinyo et al. (2011). The results were similar to the previous study that group 5 showed the highest pathogenicity compared with the other field isolates and reference strains. The advantages of using CEEs in pathogenicity study are convenience, or no raising of chickens, and determination of LD_{50} of MG organisms (Levisohn et al., 1985). However, the CEEs require free of maternally derived antibody against MG organisms. Therefore, *in ovo* inoculation model was suitable to study the pathogenicity of other Thai MG isolates in the future.

Acknowledgements

This study was carried out at the Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University and Faculty of Veterinary Science, Mahidol University. Research grants were provided by Chulalongkorn University Graduate Scholarship to commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej and the 90th Anniversary of Chulalongkorn University Fund

(Ratchadapiseksomphot Endowment Fund) and Mahidol University.

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บทคัดย่อ

การวิเคราะห์ยีนที่ก่อความรุนแรงและการก่อโรคของเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* ที่แยกได้จากประเทศไทย

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เชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* (เอ็มจี) เป็นสาเหตุของโรคทางเดินหายใจเรื้อรังในสัตว์ปีก เชื้อเอ็มจีมียีนที่ก่อความรุนแรงหลายยีนซึ่งยีนนั้นเกี่ยวข้องกับการติดเชื้อ วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อตรวจหายีนที่ก่อความรุนแรงและศึกษาการก่อโรคของเชื้อเอ็มจีที่แยกได้ในประเทศไทย การศึกษานี้มี 3 การทดลอง การทดลองที่ 1 นำเชื้อเอ็มจีจำนวน 19 สายเชื้อมาตรวจหายีนที่ก่อความรุนแรง 4 ยีน (*แอลพี แกบเอ พีวีพีเอ และเอ็มจีซี 2*) ด้วยวิธีพีซีอาร์ การทดลองที่ 2 ศึกษาการก่อโรคของเชื้อเอ็มจีในไก่กลุ่มต่าง ๆ ได้แก่ กลุ่มควบคุมลบ กลุ่มสายพันธุ์เอส 6 กลุ่มสายเชื้อที่แยกได้ 3 สายเชื้อ (เอเอชอาร์แอล 31/46 54/46 และ 58/46) และกลุ่มสายพันธุ์เอฟ ตามลำดับ ที่อายุ 21 วันจำนวน 6 กลุ่ม ๆ ละ 21 ตัว การทดลองที่ 3 ศึกษาการก่อโรคของเชื้อเอ็มจีในไข่ไก่ฟักกลุ่มต่าง ๆ เช่นเดียวกับการทดลองที่ 2 ที่อายุไข่ไก่ฟัก 8 วัน จำนวน 6 กลุ่ม ๆ ละ 15 ฟอง สำหรับการทดลองที่ 2 เมื่อไก่อายุได้ 42 วัน ทำการเจาะเลือดไก่ทั้งหมดเพื่อตรวจแอนติบอดีต่อเชื้อเอ็มจีด้วยวิธีอีไลซา ป้ายเชื้อเพื่อตรวจหาเอ็มจีด้วยวิธีพีซีอาร์ และผ่าซากเพื่อประเมินรอยโรคของถุงลมช่องอกทางมหพยาธิวิทยาและท่อลมทางจุลพยาธิวิทยา สำหรับการทดลองที่ 3 หลังจากลูกไก่ได้ฟักออกมาแล้ว ทำการแยกเลี้ยงจนถึงอายุ 7 วัน และเมื่ออายุได้ 7 วัน ทำการป้ายเชื้อลูกไก่ทั้งหมด ผ่าซากและประเมินรอยโรคดังกล่าวข้างต้น การทดลองพบว่าเชื้อเอ็มจีทั้งหมดและเชื้อเอ็มจีมาตรฐานแสดงยีนที่ก่อความรุนแรง ผลการก่อโรคของเชื้อเอ็มจีที่พบในไก่คล้ายคลึงกับที่พบในไข่ไก่ฟัก โดยเชื้อเอ็มจีเอเอชอาร์แอล 58/46 ก่อให้เกิดความรุนแรงของอาการทางคลินิก และรอยโรคของถุงลมและท่อลมมากที่สุดเมื่อเปรียบเทียบกับกลุ่มอื่น ๆ การศึกษาครั้งนี้สรุปได้ว่า เชื้อเอ็มจีที่แยกได้ในประเทศไทยสามารถก่อโรคในไก่ได้

คำสำคัญ: ไก่ ไข่ไก่ฟัก *มัยโคพลาสมา กัลลิเซพติกุม* การก่อโรค ประเทศไทย ยีนที่ก่อความรุนแรง

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