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Original Article

The Virulence of Thai Isolated *Mycoplasma gallisepticum* in Challenged Embryonated Eggs

Somsak Pakpinyo* Suwarak Wanaratana Sarawoot Mooljuntee

Abstract

This study was to investigate the virulence and pathogenicity of Thai isolated *Mycoplasma gallisepticum* (MG) inoculated into embryonated eggs and the chance of virulence study in embryonated eggs instead of experimental chickens. One hundred and twenty eight-day-old embryonated eggs were divided into 4 groups as follows. Group 1 consisting of 12 eggs served as sham negative control and was inoculated with 0.1 ml of broth into yolk sac. Group 2 (2.1, 2.2 and 2.3), 3 (3.1, 3.2 and 3.3) and 4 (4.1, 4.2 and 4.3) were inoculated with 0.1 ml of MG strains F, 6/85 and Thai isolated into yolk sacs, respectively. Each subgroup consisted of 12 eggs and the number of microorganisms differed between each subgroup, 10^8, 10^6 and 10^4 CFU/ml, respectively. Early death period (3-6 days post inoculation) and late death period (7 days and later post inoculation) were observed. When the chicks were 7 days old, blood collection was done for serology by SPA and ELISA. Then all of them were necropsied for gross thoracic airsac lesion score and microscopic tracheal lesion score, and the airsacs of all chicks were swabbed for DNA detection by PCR assay. Results revealed that the early and late death periods of all groups ranged from 0-4 and 2-7 eggs, respectively, and the number of survival chicks 0-6 days old and 7 days old were 0-3 and 1-9, respectively. The mean of thoracic airsac lesion score of dead and survival chicks was 0-1.33 without significant difference, however, significant difference was found when group 1 was compared with groups 3.2, 3.3, 4.1 and 4.2 (p<0.05). The mean of tracheal lesion score was 0.81-2.56 without significant difference. The number of positive reactors against SPA and ELISA was 0 and 1, respectively. The number of positive results against PCR assay was ranged 0-4. However, MG DNA of groups 1 and 2.3 was not observed. This study suggested that evaluation of the virulence and pathogenicity of Thai isolated MG could cause a lesion of thoracic airsac, and embryonated eggs could be used instead of experimental chickens in virulence study.

Keywords: antibody, embryonated eggs, *Mycoplasma gallisepticum*, PCR, thoracic airsac, tracheal lesion score

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บทคัดย่อ

ความรุนแรงของเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* สายพันธุ์ที่แยกได้ในประเทศไทยโดยใช้ไข่ไก่ฟัก

สมศักดิ์ ภัคภิญโญ [*] สุวรักษ์ วรรณรัตน์ สราวุธ มูลจันที

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาความรุนแรงและพยาธิสภาพของเชื้อ *มัยโคพลาสมา กัลลิเซพติกุม* (เอ็มจี) สายพันธุ์ที่แยกได้ในประเทศไทยในไข่ไก่ฟักและความเป็นไปได้ของการนำไข่ไก่ฟัก มาศึกษาความรุนแรงของการได้รับเชื้อเอ็มจีแทนตัวไก่ทดลอง ทำการแบ่งไข่ไก่ฟักอายุ 8 วัน จำนวน 120 ฟอง เป็น 4 กลุ่ม ดังนี้ กลุ่ม 1 กลุ่มควบคุมลบ (ฉีดอาหารเลี้ยงเชื้อเข้าไปที่ถุงไข่แดง 0.1 มล.) จำนวน 12 ฟอง กลุ่ม 2 (2.1, 2.2 และ 2.3), 3 (3.1, 3.2 และ 3.3) และ 4 (4.1, 4.2 และ 4.3) ทำการฉีดเชื้อเอ็มจีเสตรน เอฟ, 6/85 และ ของไทยเข้าไปที่ถุงไข่แดง (yolk sac) 0.1 มล. ตามลำดับ โดยกลุ่มย่อยของแต่ละกลุ่มนั้นมีจำนวน 12 ฟอง และปริมาณเชื้อที่ได้รับลดลง 10^8, 10^6 และ 10^4 ซีเอฟยู/มล. ตามลำดับ สังเกตการตายของคัพภะช่วงแรก (3-6 วันหลังรับเชื้อ) และช่วงท้าย (7 วันหลังรับเชื้อเป็นต้นไป) เมื่อไก่อายุ 7 วัน เจาะเลือดเพื่อตรวจหาแอนติบอดีด้วยวิธี เอสพีเอ และอีไลซา จากนั้นผ่าซากสังเกตคะแนนรอยโรคของถุงลมช่องอกด้วยตาเปล่า และท่อลมด้วยจุลพยาธิวิทยา พร้อมทั้งป้ายเชื้อที่บริเวณถุงลมเพื่อตรวจหาสารพันธุกรรมของเชื้อเอ็มจี ด้วยวิธีพีซีอาร์ ผลพบว่า ทุกกลุ่มพบจำนวนไข่ฟักที่ตายช่วงแรก ระหว่าง 0–4 ฟอง จำนวนไข่ฟักที่ตายช่วงท้าย ระหว่าง 2–7 ฟอง และพบว่า ทุกกลุ่มพบจำนวนลูกไก่ที่ฟักออกมาแล้วรอดชีวิตช่วงก่อน 6 วันแรก และอยู่รอดครบ 7 วัน ระหว่าง 0–3 ตัว และ 1–9 ตัว ตามลำดับ คะแนนรอยโรคของถุงลมช่องอกด้วยตาเปล่าของคัพภะที่ตายและลูกไก่ที่รอดชีวิต มีค่าเฉลี่ย ระหว่าง 0–1.33 โดยไม่พบความแตกต่างอย่างมีนัยสำคัญ แต่หากนำกลุ่มทดลองมาเปรียบเทียบกับกลุ่ม 1 พบว่า กลุ่ม 3.2, 3.3, 4.1 และ 4.2 พบความแตกต่างอย่างมีนัยสำคัญ ($p<0.05$) ส่วนการประเมินรอยโรคของท่อลมทางจุลพยาธิวิทยา ค่าเฉลี่ย ระหว่าง 0.81–2.56 โดยไม่พบความแตกต่างอย่างมีนัยสำคัญ จำนวนตัวอย่างที่ให้ผลบวกด้วยวิธีทางเอสพีเอและอีไลซา คือ 0 และ 1 ตัวอย่าง ตามลำดับ และผลบวกด้วยวิธีพีซีอาร์ คือ 0–4 ตัวอย่าง ซึ่งกลุ่ม 1 และ 2.3 นั้นไม่พบสารพันธุกรรมของเชื้อเอ็มจี จากผลการศึกษาพบว่าเชื้อเอ็มจีสายพันธุ์ที่แยกได้ในประเทศไทยสามารถก่อให้เกิดรอยโรคของถุงลมช่องอกได้และมีความเป็นไปได้ที่จะนำไข่ไก่ฟักมาศึกษาความรุนแรงของเชื้อเอ็มจีแทนไก่ทดลอง

คำสำคัญ: แอนติบอดี ไข่ไก่ฟัก *มัยโคพลาสมา กัลลิเซพติกุม* พีซีอาร์ คะแนนรอยโรคถุงลมช่องอกและท่อลม

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Introduction

Mycoplasma gallisepticum (MG) infection is known as a chronic respiratory disease (CRD) in avian species (Kleven, 1998; Ley, 2008). Chickens, turkeys, quails, parrots, pheasants, pigeons, and peacocks are the natural hosts of MG infection (Yoder, 1972). The mortality rate is low unless a secondary microorganism infection is present. MG infection causes sneezing, conjunctivitis, airsacculitis, and decreased egg production in affected birds (Ley, 2008). MG organisms of infected birds can be transmitted to the other birds via direct contact, which is, horizontal transmission. In addition, affected breeders can spread MG organisms through their progeny which is called "vertical transmission" (Jordan, 1996).

There are several diagnostic methods, clinical signs, histopathology, MG detection and MG serology, all of which are widely used in MG diagnosis (Kleven, 1998). MG detection including MG culture and isolation and MG polymerase chain reaction (PCR) testing has been used in most MG laboratories (Ley, 2008).

Economic losses due to the decrease in egg production in breeders have been estimated at about 21 eggs/bird, or over US$ 100 millions per year for the US poultry industry (Mohammed et al., 1987). Furthermore, their progeny show decreased feed efficiency, high conversion rate, poor carcass quality, and economic losses due to prevention and treatment costs (Ley, 2008). Therefore, MG infection in chickens has been considered as one of the most pathogenic and economic mycoplasma microorganisms of poultry (Ley, 2008). In addition, MG infection has been a great concern in the poultry industry worldwide (Ley, 2008), including Thailand (Pakpinyo and Sasipreeyajan, 2007; Pakpinyo et al., 2008). There are several MG isolates identified in Thai poultry farms including broiler, layers and broiler breeder farms (Pakpinyo and Saipreeyajan, 2007). Some isolates cause the respiratory signs previously

described, whereas some isolates do not show any clinical respiratory signs in chickens; which is similar to the study of Ley (2008) describing that isolates and strains of MG have various relative pathogenicities. Hence, further studies of pathogenecity and virulence should be conducted in order to determine precisely each type of the Thai isolates.

The pathogenicity of MG isolates and/or strains (certain isolates) can be determined by challenging studies in chickens. Several experimental studies chose to investigate the pathogenicity or virulence of mycoplasma in embryonated chicken eggs (Power and Jordan, 1973; Bradbury and McCarthy, 1983; Levisohn et al., 1985). Levisohn et al. (1985) determined the pathogenicity of virulent strain of MG in embryonated chicken eggs by the numbers of MG organisms. The objectives of this study were to evaluate the pathogenicity of Thai isolated MG inoculated into embryonated chicken eggs, the effects on embryonated chicken eggs and hatched chicks, and the possibility to use embryonated chicken eggs instead of chickens for MG experimental study.

Materials and Methods

Fertile chicken eggs: One hundred and forty fertile chicken eggs were provided by commercial broiler breeder flock free of mycoplasma infection. The egg shells were cleaned and sanitized with 70% ethyl alcohol. After cleaning and sanitizing, these fertile eggs were placed in the egg incubators for 8 days. At the eighth day of incubation, all eggs were candled to observe alive embryonated eggs. For the infertile, unhealthy or dead embryonated eggs, the egg yolks were swabbed to detect DNA of MG and *M. synoviae* (MS) by polymerase chain reaction (PCR).

MG inoculums: MG organisms were propagated from cultured broth stored at -80°C at Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University. MG strains including F and 6/85 (derived from vaccine strains), and Thai isolated were cultured, propagated, and diluted to make various amount for inoculation in eggs of different groups and subtypes.

Experimental designs: Twelve healthy embryonated eggs were inoculated with 0.1 ml of Frey's broth medium via yolk sac route, serving as Group 1 (sham inoculated control). One hundred and eight healthy embryonated eggs were equally divided into 3 groups, each group consisting of 3 subgroups. Group 2, 3 and 4 were inoculated with 0.1 ml of various strains of MG organisms including F, 6/85 and Thai isolated, respectively. Each subgroup was defined as 1, 2 and 3 and had different doses of inocula, which were 10^8, 10^6 and 10^4 colony forming unit (CFU)/ml, respectively. Each dilution of MG inoculum was injected into yolk sacs. The inoculated eggs were identified and placed into the 4 setters depending on the strains of MG and sham inoculated control. All eggs were candled once a day and observed as alive or dead embryos. At 18 days old, all live eggs were transferred to the hatchery tray. The dead embryonated eggs were necropsied to observe gross lesions including hemorrhage and size and swabbed (if possible) at the yolk sac membrane or thoracic airsac to determine the presence of the MG DNA by polymerase chain reaction (PCR) technique. After hatching, chickens of each subgroup were separately raised in card boxes and provided with feed and water *ad libitum*. All birds were observed for respiratory signs including respiratory rales, sneezing, nasal and ocular discharge. At 7 days old, all chickens were bled for MG serology including serum plate agglutination (SPA) and ELISA, then euthanized and necropsied. The necropsied birds were blindly evaluated for gross thoracic airsac lesion score, histopathological tracheal lesion score and simultaneously swabbed at the left side of the thoracic airsac to determine the presence of the MG DNA by PCR technique.

Classification of dead embryonated eggs: The dead embryonated eggs were observed as an early or late death (modified from Levisohn et al., 1985). The early or late death was the embryo found dead during 3-6 days or 7-11 days post inoculation, respectively.

Yolk sac inoculation procedure: Yolk sac inoculation followed the procedure reviewed by Senne (1998). Briefly, an eight-day-old healthy embryonated egg was candled, the air cell and embryo were located, the egg shell was sanitized, a needle was inserted through the top and center of the air cell side up, and the pore was closed with candle wax. The inoculated egg was discarded if the embryo died prior to 48 hours.

Airsac lesion score: The airsac lesion score was grossly evaluated by the following criteria (Kleven et al., 1972): 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 the same as 3, but 2 or more airsacs are found.

Tracheal lesion score: The tracheal lesion score was microscopically evaluated as the following criteria (Yagihashi and Tajima, 1986): 0: No significant changes are observed., 1: Small aggregate 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.

MG serology

SPA procedure: Fresh sera were tested with MG antigen (Nobilis®, Intervet International BV, Holland) following the manufacturer's instructions. Briefly, thirty µl of serum were mixed with 30 µl of antigen then incubated at room temperature for 1-2 min before the result could be observed. Negative and positive sera were also included in each test. Sera were then stored at -20°C for ELISA determination.

ELISA: Frozen sera were completely thawed at room temperature (25°C) before testing. All procedures were done at room temperature. Sera were tested with commercial test kits, ProFLOK® (Synbiotics Corporation, USA) following the manufacturers' directions. Briefly, diluted sera were added into MG antigen-coated plate, incubated, washed then peroxidase-labeled anti-chicken antibody (conjugated antibody) was added. After incubation, the plate was washed, then a substrate was added, and finally, a stop solution was added. The plate was read in an ELISA reader at 405 nanometer manufactured by Labsystems Multiskan MG Type 352, Finland. The optical density of the negative and positive controls and the samples was calculated, then interpreted according to the manufacturers' recommendation. For the interpretation of ELISA, titer levels 0-148, 149-743, and equal or higher than 744 are negative, suspicious and positive reactors, respectively.

MG DNA detection

PCR procedure: The broth sample was individually determined in this study. This method was described by Lauerman (1998). Briefly, the broth was extracted for DNA template by centrifugation at 15,000xg, washed with distilled water, followed by dilute pellet with distilled water, boiled for 10 min, and then placed at -20°C for 10 min, ending with centrifugation and collection of the supernatant at -20°C until use. For PCR mixture in 50 µl volume, KCl 500 mM, Tris-HCl (pH 8.3) 100 mM, dNTP (Fermentas) 1 mM, primer F (5′ GAGCTAATCTGTAAAGTTGGTC 3′) and primer R (5′ GCTTCCTTGCGGTTAGCAAC 3′) (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 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 and followed by 72°C for 5 min. The 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: The gross airsac and histopathological tracheal lesion scores were determined by using Chi square test at $p < 0.05$. Significant difference between the sham negative control group and the treatment groups was analyzed by Mann-Whitney U test. All statistical analyses were tested by SPSS for Windows version 17.0.

Results

For the infertile, unhealthy or dead embryonated eggs prior to 8 days of incubation, the egg yolks could not detect the MG and MS DNA. This study revealed that the early and late deaths were found ranging from 0-4 and 2-7 embryonated eggs, respectively. The survival chicks prior to 7 days old and at 7 days old were 0-3 and 1-9 birds, respectively, nothing that group 2.3 had the lowest survival at 7 days old. The dose of inoculum of all strains of MG did not apparently affect the survival chicks (Table 1).

The average of gross thoracic airsac lesion score and histopathological tracheal lesion score of the dead embryos and survival chicks ranged from 0-1.33 and 0.81-2.56, respectively, without significant difference; however, a significant difference was observed only in the gross airsac lesion score between group 1 and the treatment groups including 3.2, 3.3, 4.1 and 4.2 (Table 2).

The numbers of positive samples against SPA test, ELISA and MG PCR were 0, 1 and 0-4, respectively. MG DNA could not be detected in group 1 and 2.3 (Table 3). In addition, MS DNA was not found in the survival chicks at 7 days old of all groups.

Table 1 Numbers of embryonated egg deaths during early and late death period and numbers of dead chicks prior to 7 days old and survival chicks at 7 days old

Group	Number of embryonated egg deaths		Number of chick deaths prior to 7 days old	Number of survival chicks at 7 days old
	Early death	Late death		
1 (Neg.)	1	2	0	9
2.1 (F,10^7)	4	4	2	2
2.2 (F,10^5)	1	6	0	5
2.3 (F,10^3)	4	7	0	1
3.1 (6/85,10^7)	1	4	0	7
3.2 (6/85,10^5)	1	3	0	8
3.3*(6/85,10^3)	1	4	0	6
4.1 (Thai,10^7)	0	2	1	9
4.2 (Thai,10^5)	1	2	3	6
4.3 (Thai,10^3)	3	2	1	6

*One embryonated eggs was discarded due to death before 48 hours; the total numbers was 11 embryonated eggs.

Table 2: Blind evaluation of gross thoracic airsac and histopathological tracheal lesion scores (n: numbers of pooled samples of dead embryos and survival chicks).

Group	Thoracic airsac lesion score	Tracheal lesion score
1	0±0 (n=9)	1.63±0.62 (n=4)
2.1	1.13±1.44 (n=4)	2.38±0.52 (n=2)
2.2	0.80±1.79 (n=5)	2.56±0.51 (n=4)
2.3	0 (n=1)	2±0 (n=1)
3.1	0.40±0.52 (n=10)	1.69±0.60 (n=4)
3.2	1.09±1.58 (n=11)	0.81±0.54 (n=4)
3.3	1.11±1.36 (n=9)	1.38±0.50 (n=4)
4.1	1.33±0.98 (n=12)	1.44±0.51 (n=4)
4.2	0.60±0.70 (n=10)	1.13±0.62 (n=4)
4.3	0.71±1.50 (n=7)	1.38±0.62 (n=4)

Table 3: Numbers of positive samples against SPA test, ELISA and MG DNA positive samples by PCR

Group	Numbers of positive reactors		Numbers of MG DNA positive samples by PCR
	SPA	ELISA	
1*	0/9	0/9	0/9
2.1	0/2	0/2	1/4
2.2*	0/5	1/5	4/5
2.3*	0/1	0/1	0/1
3.1	0/7	0/7	1/10
3.2	0/8	0/8	1/11
3.3	0/6	0/6	1/9
4.1	0/8	0/8	4/12
4.2	0/5	0/5	2/10
4.3	0/6	0/6	2/7

*MG PCR was tested only on survival chicks at 7 days old

Discussion

This study revealed that all strains and Thai isolated of MG caused the death of embryonated eggs and the lesions of the thoracic airsac including presence of the blood vessels, cloudy, thickening and caseous mass. In addition, for the survival chicks at 7 days old, the gross thoracic airsac and histopathological tracheal lesion scores did not depend on the dose of inoculum.

The study found that the F strain caused the highest embryo mortality, followed by 6/85 strain and Thai isolated, respectively. Generally, the F strain is more virulent vaccine compared with ts-11 or 6/85 strains (Whithear, 1996). However, the virulence of MG Thai isolated was similar to that of 6/85 in cases that the data of survival chicks were considered. Three deaths of embryonated eggs of the sham negative control were found without remarkable lesion or bacterial growth suggesting the normal death of embryos.

The MG Thai isolated used in this study showed less pathogenicity compared with in vivo pathogenicity (Pakpinyo, 2005). From our previous in vivo study, the MG-inoculated chicks showed severe gross thoracic airsacs and histopathological tracheal lesion scores, and high mortality. Interestingly, this study had a similar result to Levisohn et al. (1985) in that there is no or only a little relation between *in ovo* and *in vivo* pathogenicity study. Furthermore, Levisohn et al. (1985) found that the numbers of embryo mortality did not have a correlation with the dose of MG inoculation, which corresponded to our study. The reason of low dose of MG inoculation causing the higher numbers of embryos deaths during the late period was possibly due to the enormous growth of MG microorganisms in the yolk even though non virulent strain leading to embryo deaths (Lin and Kleven, 1984; reviewed by Levisohn et al., 1985).

The serological results of the treatment groups found only 1 positive reactor against ELISA out of 48 samples. This was possibly due to the early stage of immune response of hatched chick; if this study was extended, the numbers of positive reactors would surely increased. Interestingly, all 48 samples of the treatment groups were not found positive by the SPA test possibly due to what was previously described. Actually, this procedure should detect the positive reactor from this study because SPA test detects immunoglobulin (Ig) M, which is the first Ig to be formed at approximately 7-10 days after infection (Kleven, 1975; Kleven, 1981). However, almost all of the treatment groups presented the MG DNA, except group 2.3, which had only 1 survival chick left to be performed by PCR. For the positive MG DNA sample, the present study suggested that the gross thoracic airsac lesion scores equal or higher than 2 appeared to be positive by PCR, which was useful for the servicemen or veterinarians to diagnose MG. However, the airsacculitis can be found in other infectious diseases including *Mycoplasma synoviae*

infection (Kleven and Ferguson-Noel, 2008), colibacillosis (Barnes et al., 2008), *Ornithobacterium rhinotracheale* infection (Chin et al., 2008) etc.

The present study reveals that the evaluation of the virulence or pathogenicity of Thai isolated MG by using embryonated eggs is possible. There are several advantages of using embryonated eggs including no requirement of experimental chickens and rooms, shorter duration compared with in vivo study, uncomplicated rearing etc. Conversely, several aspects should be concerned including the quality of embryonated eggs, none of the maternal-derived antibody against MG, the adequate capacity to place eggs in the same incubator, the ambiguous classification during egg candle between dead or alive embryonated eggs etc. Therefore, the use of embryonated eggs instead of chickens is possible unless these weak aspects have not been managed.

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