SCIREA Journal of Animal Husbandry and Veterinary Medicine

http://www.scirea.org/journal/AHVM

October 22, 2016

Volume 1, Issue1, October 2016

Pathogenicity of various *Mycoplasma gallisepticum* strains in vaccinated and non-vaccinated breeders flocks' chicken embryos

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Abstract:

This study was undertaken to determine the pathogenicity of six of *Mycoplasma gallisepticum* (MG) local field strains in specific pathogen free and commercial broiler breeder chicken embryos and the appropriate viscera organs for sampling in hatched chickens for detection and isolation of *Mycoplasma gallisepticum.* Three hundred eighty (380) each embryonated eggs were grouped into three, according to reference strains (MGS6 and ts-11), field isolates and control

(uninoculated and broth inoculated) groups. The embryonated eggs were each inoculated with 0.2ml “pleuropneumonia like organism” (PPLO) broth containing 6.2 x 10 CCU/ml of reference strain or field isolate respectively, via yolk sac, at day 6 of incubation and were examined at necropsy days 7, 10, 13 and 15 postinoculation. Grossly, reference and field MG infection groups showed more significant lesion findings when compared to control groups in SPF embryonated eggs however not significant in commercial broiler embryonated eggs. Postmortem findings of inoculated embryos for both type embryos were: dwarfing, curled toes, head edema, slightly enlarged and pale liver, slightly enlarged and pale spleen and green colour liver. Histopathological lesions of embryos from MG inoculated groups of all sources revealed that there were mild to severe infiltration of inflammatory cells such as neutrophils and lymphocytes. Caseous air sac lesion score ranged from 0 to 1 for SPF embryos and 1 to 3 lesion score for commercial broiler embryos which might be due to the formation of antigen-antibodies complexes.

Key words: Mycoplasma gallisepticum, pathogenicity, Caseous airsac, Embryonated eggs

Introduction

Mycoplasma gallisepticum (MG) infection commonly causes diseases known as chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys. *Mycoplasma gallisepticum* infections are characterized by respiratory rales, coughing, gasping, nasal discharge and conjunctivitis. The clinical manifestations are always slow to develop and the disease may have a long course. *Mycoplasma gallisepticum* is capable of replicating in cell-free medium and usually stays at the epithelial surfaces of respiratory and reproductive tract (Nelson, 1935). This enables the lateral and vertical transmission to occur in chickens. The organism is relatively fragile and survives for only a few days, ranging from 2 to 16 days, outside the body of the host in the environment at a temperature of 20 ℃. However, survival may be longer when the organism is protected in exudates at lower temperature or when organism lives in yolk of eggs kept at 37 ℃ (Christensen et al., 1994).

Vertical transmission either *in ovo* or transovarian of MG is known to occur in eggs laid by naturally infected hens (Glisson et al., 1984). This appears to be important in spreading the organism from one generation to another and subsequently caused great economic losses. The lateral transmission is mainly by aerosol and probably through contact of susceptible chickens with nasal exudates, moist nostrils or watery eyes, contaminated feathers and dust. Increasing the population density increased the rate of the horizontal infection of MG (Yoder et al., 1991). Poor farming biosecurity and poor personnel practice are also important which can lead to MG infection. The outcome of MG infection is influenced by the age of the host. Young chicks are more susceptible than older chickens and eventually develop more severe disease. *Mycoplasma gallisepticum* has been known to become more virulent when the isolate is passaged in vivo. Maternal antibodies against MG have been reported in embryos and newly hatched chicks for up to 18 days of age.

Mycoplasma infection causes significant economic losses to the poultry industry. Economic losses from condemnations or downgrading of carcasses, reduced feed conversion efficiency and egg production (Yoder, 1991; Bradbury, 2001). The increased medication costs are additional factors that make this infection one of the costliest disease problems confronting commercial poultry production worldwide. In commercial broiler farms, the mortality due to airsacculitis resulting from MG ranged from 10% to 30%. Infection by MG is of particular concern, because it predisposes birds to secondary infection by *Escherichia coli*, which can lead to high carcass condemnations at processing (Yoder, 1991). The appearance of CRD in a chicken flock is dependent on other concurrent bacterial and viral infections such as *E. coli*, infectious bronchitis, infectious bursal disease and Newcastle disease viruses. Nutritional deficiency and social stress are the debilitating factors that may predispose birds to CRD. The onset of lay and vaccination programme which caused stress can increase the incidence of MG infection.

Though flock health programs such as vaccination and usage of antibiotics are practiced to prevent MG infection, the incidence of MG infections is still prevalent in Malaysia and worldwide (Yap, 2005). There is also still lack of information and reports about MG infection in different sources of eggs from breeder farms. Several breeder farms in Malaysia have complaint of caseous air sac present in the day old chicks. Caseous airsac is highly suggestive of mycoplasma infection, *E. coli* or aspergillosis infection. Aspergillosis and *E. coli* are either as a result of pre or post incubation of eggs which most probably is due to poor management.

Therefore, the objectives of the study were: (1) to determine the pathogenicity of different MG isolates in SPF chicken embryos. (2) to determine the pathogenicity of different MG isolates in commercial chicken embryos with and without history of MG outbreak.

Materials and Methods

Embryonated chicken eggs. Three hundred and eighty specific pathogen free (SPF) embryonated eggs were obtained from Veterinary Research Institute (VRI), Ipoh, Perak, Malaysia. Three hundred and eighty commercial broiler breeder embryonated eggs were obtained from local commercial company, Malaysia with history of killed MG vaccine application. The serology of the commercial breeders was shown in Figure 3. Out of 380 each embryonated eggs, 38 eggs were used for each group: 1 MG reference strain (MG-S6), 1 MG vaccine strain (ts-11), 6 MG field isolates (H21-8T, H21-11T, H24-5C, H26-9C, I-18 and I-44) and control (uninoculated and broth inoculated) eggs. All the eggs were then incubated at 37°C for 6 days. On the sixth day before inoculation, every egg was candled.

Mycoplasma strains. Reference strains and field isolates of MG were used in this study. The reference strain (MG-S6) was obtained from Veterinary Research Institute (VRI), Ipoh, Perak, Malaysia, and the vaccine strain (ts-11) was obtained from VAXSAFE MG TS-11, BIOPROPERTIES Pty Ltd. Field isolates I-18 and I-44 was isolated by Tan (2004) from apparently healthy village chickens, H21-8T was obtained from infected broiler breeder chickens, H21-11T was from infected normal chick, H24 5C was from infected broiler chickens and H26-9C was from pipped embryo. Strain MG-S6 is reported to be one of the highly pathogenic MG strains (Jordan, 1979; Power and Jordan, 1976; Zander, 1961).

Determination of Inoculum. The number of viable mycoplasma organism inoculated was determined by micro-broth dilution using "pleuropneumonia like organism" (PPLO) broth. Growth was indicated by a change in the phenol red indicator in the medium, from red to yellow. The number of viable organisms in the original cultures was determined by using the tables published by Meynell and Meynell (1970) and was expressed as the most probable number of color-changing unit (CCU)/ml. The amount of the inoculum in this study was 6.2×10^5 CCU/ml, based on the study by Lockaby et al., (1999) and O'Connor et al., (1999).

Embryo Inoculation. All 380 SPF and commercial broiler breeder embryonated eggs were labeled according to reference, field, and control groups. The egg shell was swabbed with 70% alcohol before inoculation of the organism into the yolk sac. The blood vessels of the eggs were viewed with using egg candler. These vessels may appear as nothing more than an array of faint lines, orange in color, extending from a clear halo. The embryos were within the area of the halo close to the margin of the air cell. A hole was made at the top of the shell with an egg punch. Each group of 38 embryonated eggs were inoculated via yolk sac by using 23G, 1½ inch long needle, at 6 days of incubation with approximately 6.2 X 10^5 CCU/ml of each reference and field strain isolates, respectively in 0.2ml PPLO broth. No inoculation was done on control uninoculated eggs. Embryonated eggs were maintained in incubator at 37.5℃ with 60% humidity. The eggs were candled daily; any dead embryos within 2 day post-inoculation (pi) were discarded.

Post mortem Examination of Embryos. *Mycoplasma gallisepticum*-inoculated and control embryonated eggs were examined at necropsy on days 7, 10, and 13 post-inoculation (pi). The embryos that died prior to the necropsy were kept in the refrigerator at 4℃ for 24 hours and examined the day after. Amnioallantoic fluid was cultured on the PPLO broth for mycoplasma isolation and on blood agar to verify absence of bacterial contamination. During necropsy, embryos were weighed and the body cavity was incised. The organs such as liver, heart, lungs, spleen, bursa, trachea, kidney, gizzard, yolk sac and chorioallantoic membrane (CAM) were examined before fixing the whole embryo in 40% neutral buffered formalin. All organs were embedded in paraffin. Tissue sections were cut at 3-5µm and stained with haematoxylin and eosin (HE) for observation under the light microscopy. Swabs were taken from choanal cleft, trachea, airsac and yolk sac for culturing on the PPLO agar for mycoplasma isolation and polymerase chain reaction (PCR). For the eggs that either hatched completely or not hatched (dead in shell or pipped embryo) after 21 days of incubation, post mortem was carried out on the chicks from reference, field, and control groups. Gross lesions were examined and samples were fixed using 40% formalin before embedding in paraffin and stained with HE for observation under light microscopy.

Lesion Scoring. Airsacs lesions were graded based on its severity subjectively; 1+ indicates mild infection, 2+ indicates moderate infection and 3+ indicates severe infection. Infection or non-infection of the tissue was determined based on the infiltration of inflammatory cells. Lesion

scoring was made and classified as below: 0; no inflammatory cells infiltration, 1: mild inflammatory cells infiltration, 2: moderate inflammatory cells infiltration and 3: severe inflammatory cells infiltration.

Verification of Mycoplasma Strain. On days 7, 10, 13 and 15 pi, choanal cleft, trachea, airsac, yolk sac and amnion-allantoic swabs were collected from all the eggs that need to be necropsied, inoculated back to PPLO agar before being placed in the mixture of plain PPLO broth and glycerol. They were used for PCR. This was done to verify the presence of MG in the inoculated eggs. Genomic DNA and RNA (total nucleic acid) were extracted using a commercially available kit, MasterPure™ Complete DNA Purification Kit (Epicentre® Biotechnologies), by following method recommended by the manufacturer. The conventional PCR procedures were performed according to method described by Marois et al., (2000) with some modification. The primer used in this study was designed to bind the 16S rRNA genes and amplify a 186bp DNA fragment and the sequence is MG-14 F 5' GAGCTAATCTGTAAAGTTGGT 3' and MG-13 R 5' GCTTCCTTGCGGTTAGCAAC 3'. The reaction volume was set up in a 25 μl reaction mixture. The reaction procedure consisted of an initial denaturation step at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute and extension at 72°C for 1 minute 30 seconds, and ended with 1 cycle of final extension at 72°C for 10 minutes. PCR amplified DNA fragments were separated by using 1.5 % agarose gel electrophoresis as the detection of genomic DNA. The size of the amplified product was compared using a 100bp plus DNA ladder (Vivantis®, Malaysia).

Statistical Analysis. This statistical analysis was done to determine significance of MG infection on weight and organs of embryos from reference, vaccine and field groups and to compare pathogenicity of MG infection in SPF embryonated eggs between reference, vaccine and field groups at days 7, 10, 13, and 15 post-inoculation based on blocked time 2 way anova Test.

Results

Mean Weight of Chicken Embryos. Mean of SPF embryos weight (Figure 1) from reference and field groups were lower ($p < 0.05$) than the mean of embryos weight from control group at day 10 and 13 pi. At day 7 and 15 pi, mean weight of control eggs is not significantly different

($p > 0.05$) from mean weight of MG reference group and mean weight of field group. At day 10 and 13 pi, mean weight of control eggs is significantly different from the mean weight of reference group and mean weight of field group. At day 7 pi, weight of embryos for control (uninoculated) was 7.8±0.8g, control (broth) was 7.4±1.6g, reference MG-S6 was 7.5±0.5g, vaccine ts-11 was 8.1±0.7g and field isolates ranged from 6.8±1.1g - 8.3±1.3g. At day 10 pi, weight of embryos for control (uninoculated) was 22.2±1.8g, control (broth) was 22.2±2.2g, reference MG-S6 was 19.0±2.9g, vaccine ts-11 was 17.9±4.3g and field isolates ranged from 8.2±1.1g - 19.8±3.1g. At day 13 pi, weight of embryos for control (uninoculated) was 30.6±2.7g, control (broth) was 29.7±5.7g, reference MG-S6 was 21.8±6.0g, vaccine ts-11 was 25.3±5.9g and field isolates ranged from 16.7±1.2g - 25.7±4.5g. At day 15 pi, weight of embryos for control (uninoculated) was 41.4±3.9g, control (broth) was 40.1±2.7g, reference MG-S6 was 42.8±2.3g, vaccine ts-11 was 44.1±3.6g and field isolates ranged from 39.4±5.8g - 43.4±3.6g.

Mean of commercial broiler breeder embryos weight (Figure 2) from reference, vaccine and field groups were almost the same ($p > 0.05$) with the mean of embryos weight from control group for all the sampling. Mean weight of control eggs was not significantly different ($p > 0.05$) from mean weight of MG reference, vaccine and field groups for all time of samplings. At day 7 pi, weight of embryos for control (uninoculated) was 16.9±2.2g, control (broth) was 16.2±2.3g, reference MG-S6 was 16.4±2.3g, vaccine ts-11 was 13.7±4.7g and field isolates ranged from 13.3±3.4g - 16.4±2.0g. At day 10 pi, weight of embryos for control (uninoculated) was 27.3±1.1g, control (broth) was 27.5±4.4g, reference MG-S6 was 26.9±2.7g, vaccine ts-11 was 27.7±4.1g and field isolates ranged from 25.4±5.5g - 29.1±1.9g. At day 13 pi, weight of embryos for control (uninoculated) was 50.1±0.5g, control (broth) was 45.2±4.2g, reference MG-S6 was 45.9±5.6g, vaccine ts-11 was 48.0±3.3g and field isolates ranged from 43.0±2.1g - 49.1±3.5g. At day 15 pi, weight of embryos for control (uninoculated) was 43.1±3.9g, control (broth) was 43.2±2.8g, reference MG-S6 was 41.7±4.0g, vaccine ts-11 was 46.0±5.8g and field isolates ranged from 42.9±2.4g - 47.2±6.0g.

Gross Lesions. The gross lesions observed from reference, vaccine and field groups were based on comparative lesions from control groups at day 7, 10, 13 and 15pi. They were six gross lesions found at all time of sampling such as embryo head edema and pale in appearance, slightly enlarged and pale liver, slightly enlarged and green liver, slightly enlarged and pale spleen, curled toes, caseous airsac and dwarfing in SPF embryos groups. In commercial broiler breeder embryos

groups, they were five gross lesions found at all time of sampling such as slightly enlarged and pale liver, slightly enlarged and green liver, white spotted liver, curled toes, and dwarfing.

Caseous Airsac. Caseous airsac only can be observed at day 15 pi. At day 15 pi, eggs from all groups were allowed to hatch after 21 days of incubation. Air sacs of day old chicks or embryos were examined. In SPF embryos groups, no caseous airsac was detected in the control, broth, H21-8T and H24-5C groups. However, there were 30 % of the embryos from MG-S6 and H21-11T, 20% of embryos from ts-11, I-18 and H26-9C, 10% of embryos from I-44 had caseous airsac. The severity of caseous air sac was only mild (+1). In the commercial broiler breeder embryos groups, no caseous air sac was detected in the control, broth and H21-11T groups. However, there were 10 % of the embryos from MG-S6, 20% of embryos from I-44, 40% of embryos from H26-9C, 50% of embryos from ts-11, 60% of embryos from H21-8T, 70% of embryos from H24-5C and 80% of embryos from I-18 had caseous airsac. The severity of caseous airsac ranged from mild (1+) to severe (3+). A total 19 out of 26 from breeder source without any history of MG outbreak before had caseous airsac. A total 14 out of 32 from breeder source with history of MG outbreak before had caseous airsac. Mean 73% of source without MG outbreak before compared to 44% from source with MG outbreak before.

Other lesions. Green liver that were observed from MG inoculated group were based on comparative lesion from control and broth inoculated groups at day 7, 10 and 13 pi for SPF embryos groups which 11%, 11% and 32% respectively; 7 and 10 pi for commercial broiler breeder embryos groups which 6% for both pi. No green liver was observed at day 15 pi for both SPF and commercial embryos groups. Dwarfing, head edema and pale, curled toes, liver slightly enlarged and pale, and spleen slightly enlarged and pale were other gross lesions found in the MG inoculated group embryos for both SPF and commercial broiler breeder embryos groups. However this finding was not obviously different.

Hatchability. The eggs were allowed to hatch after 21 days and were classified into 3 groups, which are: fully hatched, pipped embryos and dead-in-shell embryos. All of the SPF and commercial breeder broiler eggs from control and broth groups were able to hatch. For SPF, all the chicks that hatch from the control and broth inoculated groups were normal chicks. Eggs from group MG-S6, H21-8T, H21-11T and H24-5C were not able to hatch and classified as pipped embryos and dead-in-shells. Only few eggs from group ts-11, I-18, I-44 and H26-9C were

hatched as poor quality chick, most of other eggs were unable to hatch (Table 1). For the commercial broiler breeder embryos, all the chicks that hatch from the control and broth group were normal chicks except one from the hatch chick was poor quality chick. All eggs from group MGS6 and I-44 were able to hatch. Six of 10 from MGS6 group were normal chicks and the remaining were poor quality, 8 of 10 from I-44 group were normal chicks and the other were poor quality chicks. The hatchability for strain ts-11, I-18, H21-8T, H21-11T, H24-5C and H26-9C were ranged from 10%-40%. All hatched chicks from group ts-11, I-18, H21-8T and H21-11T were normal chicks. Two of 3 hatched from H24-5C and 2 of 4 hatched chicks from H26-9C were poor quality chicks. All groups had dead-in-shell category (Table 2).

Lesion Scoring. At day 7, 10, 13 and 15 pi of the SPF embryonated eggs, the histological lesions from reference, vaccine and field groups mainly revealed mild to moderate infiltration of neutrophils and lymphocytes in liver, trachea, kidney, CAM, lungs, gizzard, heart, spleen, bursa, and yolk sac. At day 7pi, histopathological lesions of organs in reference and vaccine groups were more severely affected ($p < 0.05$) than field groups. Histopathological lesion scoring in reference and vaccine groups ranged from 0.3 to 1.9 while lesion scoring in field groups ranged from 0.0 to 2.0. Lesion scoring of organs in reference and vaccine groups were higher ($p < 0.05$) than field groups except lesion scoring of yolk sac and bursa which showed that the scores in field groups were higher ($p < 0.05$) than in reference and vaccine groups. At 10 day pi, histopathological lesion scoring of liver, trachea, CAM, kidney, heart, spleen and yolk sac in reference and vaccine groups were more severely affected ($p < 0.05$) than field groups. There were marked inflammatory cells infiltration in gizzard, which was from field groups. Lesions scoring in lung and bursa from reference, vaccine and field groups were almost similar ($p > 0.05$). At 13 day pi, histopathological lesion scoring of liver and trachea in reference and vaccine groups were more severely affected ($p < 0.05$) than field groups. There were almost similar lesion score ($p > 0.05$) observed in CAM, lung, gizzard, kidney, bursa, heart, yolk sac and spleen from reference, vaccine and field groups. The histopathological results at day 15 pi which consisted of pipped embryos, dead in shell and poor quality chicks that lesion scoring of organs such as liver, trachea, kidney, bursa, lungs, gizzard, heart, and spleen in reference and vaccine groups were more severely affected ($p < 0.05$) than field groups. There were almost similar lesion score ($p > 0.05$) in CAM and yolk sac from reference, vaccine and field groups. Organs such as CAM, liver, trachea, kidney, bursa, lungs, spleen, and gizzard showed significance ($p < 0.05$) when

embryonated eggs were infected by MG at day 7, 10, 13, and 15 pi based on blocked time 2 way anova. Result revealed that reference and vaccine strains of MG infection in SPF embryonated eggs were more pathogenic when compared to field isolates of MG infection in SPF embryonated eggs.

For the commercial broiler breeder embryos, the histological lesions from reference, vaccine and field groups mainly revealed mild infiltration of neutrophils and lymphocytes in liver, trachea, kidney, CAM, lungs, gizzard, heart, spleen, bursa, and yolk sac at day 7 pi. The results showed that histopathological lesion scoring of organs in reference and vaccine groups were almost similar ($p > 0.05$) to field groups. At day 10 pi, the histological lesions from reference, vaccine and field groups mainly revealed mild to moderate infiltration of neutrophils and lymphocytes in yolk sac, trachea and CAM. Histopathological lesion scoring of yolk sac, trachea and CAM reference and vaccine groups were more severely affected ($p < 0.05$) than field groups. Lesions scoring in liver, kidney, heart, spleen, gizzard, lung and bursa from reference, vaccine and field groups were almost similar ($p > 0.05$). At day 13 pi, the histological lesions from reference, vaccine and field groups mainly revealed mild to moderate infiltration of neutrophils and lymphocytes in liver, trachea, kidney, CAM, lungs, gizzard, heart, spleen, bursa, and yolk sac. The histopathological lesion scoring of CAM, liver, trachea, lung, gizzard and yolk sac in reference and vaccine groups were more severely affected ($p < 0.05$) than field groups. There were almost similar lesion score ($p > 0.05$) observed in kidney, bursa, heart, and spleen from reference, vaccine and field groups. The histopathological results day 15 pi which consisted of pipped embryos, poor quality chicks and normal chicks, revealed mild to severe infiltration of neutrophils and lymphocytes in liver, trachea, kidney, CAM, lungs, gizzard, heart, spleen, bursa, and yolk sac which the result showed that histopathological lesion scoring of organs such as trachea, kidney, and heart in reference and vaccine groups were more severely affected ($p < 0.05$) than field groups. There were almost similar lesion score ($p > 0.05$) in CAM, liver, bursa, lung, gizzard, spleen and yolk sac from reference, vaccine and field groups. The statistical analysis of organs such as CAM, liver, trachea, kidney, bursa, lungs, spleen, yolk sac, gizzard and gizzard showed no significance ($p > 0.05$) when embryonated eggs were infected by MG at day 7 and 10 pi. Significant results ($p < 0.05$) were obtained when embryonated eggs were infected by MG at day 13 and 15 pi. Besides that, statistical analysis result revealed that reference and vaccine

strains of MG infection in commercial embryonated eggs were almost similarly pathogenic ($p > 0.05$) when compared to field isolates of MG infection in commercial embryonated eggs.

Reisolation of MG. *Mycoplasma gallisepticum* was able to be reisolated from all 5 sites of samplings (choanal cleft, airsac, trachea, yolk, and CAM) from chick embryos and chicks (normal or poor quality chicks) that had no caseous airsac or caseous airsac score of 1. Trachea and airsac were the best sites for the chick embryos and chicks for detection of MG in this study. MG could not be reisolated from the chick embryos and chicks that had caseous airsac score of 2 and 3. All MG inoculated samples were positive for MG detected by PCR.

Pattern of Pathogenicity. I44 and I-18 strains of MG have a similar pattern of pathogenicity in that they are highly pathogenic. Whereas, ts-11 (vaccine strain), H21 8T, H21 11T, H24 5C and H26 9C have similar pattern of pathogenicity characterized by their less pathogenicity in embryos. MGS6, I44 and I-18 strains caused early embryonic death compared to ts-11, H21 8T, H21 11T, H24 5C and H26 9C strains that caused embryo mortality during later stages of incubation.

Discussion

Avian mycoplasma serotypes are found to differ in their potential for producing embryo mortality, with most of the strains of MG being pathogenic for chicken embryos (Yoder, 1964). The cultural history of the MG strain also correlates with the embryo mortality (Roberts and Olesiuk, 1966). Pathogenic MG strains cause a high embryo mortality, but it may be possible that the in ovo virulence is enhanced by egg adaptation (Levisohn et al., 1985). Some non-pathogenic or less pathogenic MG strains may cause embryo mortality but this may be attributable to the massive multiplication rather than the actual virulence of a particular strain and this requires further study (Levisohn et al., 1985). Besides MG, some avian mycoplasmas that have been tested in chicken or in turkey embryos include *Mycoplasma iowae, Mycoplasma synoviae, Mycoplasma meleagridis, amd Mycoplasma gallinarum* (Kleven, 1998).

Based on a large number of MG isolates from different countries, flocks and hosts has been noticed to show a great variation in biological properties, such as virulence, infectivity, tissue proclivity (cerebral arteriotropism) with different pathological profiles and specificity for cloacal, joint or eye infection (Jordan 1979, Yoder 1991, Yogev et al., 1988, Yogev et al., 1989) resulting

in a very great variations in epidemiological, clinical and pathomorphological aspects. Some MG strains has reported for causing severe air sac lesions or substantial loss of egg production over a five-week period after injection into the abdominal air sacs of chickens as well as on certain MG strains which did not produce gross lesions or loss of egg production (Soeripto et al., 1989). The disease causes a 10% to 20% decrease in egg production (nearly 16 fewer eggs per hen) and a 5% to 10% increase in embryo mortality in breeders and layers. Some developing embryos may be killed but enough infected chicks hatch to allow for transmission of the disease within the flock. It is important to mention that the longest survival time was observed in egg materials (in allantoic fluid: 3 weeks at 5 ℃, 4 days in the incubator, 6 days at room temperature; in egg yolk: 18 weeks at 37 ℃ or 6 weeks at 20 ℃). Therefore, egg debris in incubators is essential in spreading infection. It is also interesting to note that MG can survive for one to two days on human hair and skin. So, people working with infected flocks can also act as MG carriers (Yoder 1991).

Some MG strains spread very quickly and were inducing a serological response in contact with infected chickens as little as four weeks. While other strains spread very slowly and producing a serological reaction after sixteen weeks. Differences were also observed in egg transmission. Consequently, the disease may range from very mild to very severe; it can spread slowly or very quickly, and it can either be diagnosed easily by isolation of typical strains and/or demonstration of a strong serological response, or the diagnostic procedure may be very difficult because of the isolation of so-called variant (atypical) strains, or the development of a very poor serological response (Yoder 1986). Certain isolates of MG have become more commonly known by their designation: the A5969 strain S6 was isolated from the brain of a turkey with infectious sinusitis became a standard strain for antigen preparation; strain R, which was isolated from a chicken with airsacculitis is used for challenge and bacterin production and strain F is commonly used in live vaccination programmes (Kleven et al, 1988, Kleven et al., 1990, Ley et al., 1993, Yoder 1991, Yogev et al., 1988, Yogev et al., 1989, Yogev et al., 1994).

Significant difference of mean embryo weight was only found in SPF embryonated eggs. Mean of embryos weight from MG inoculated group of commercial embryos were not significantly different from the mean of weight of embryos from control and broth inoculated groups. The SPF eggs inoculated with MG totally failed to hatch. The commercial broiler breeder eggs inoculated with MG that hatched ranged 10% to 100%. This indicates that there is still a possibility for the

eggs to hatch if MG is present in the eggs. There were 60% to 90% pipped embryos from MG inoculated group of commercial broiler breeder eggs. None of the MG inoculated embryos was dead in shell.

Dwarfing, generalized edema, liver necrosis, and enlarged spleen are most typical lesions in the embryos after inoculation or infection by MG (Kleven, 1998). The organism reaches its highest concentrations in the yolk sac, yolk, and chorioallantoic membrane prior to embryo death. Studies showed that MG strains varied in their in ovo pathogenicity and there is no correlation between in ovo pathogenicity and in-vivo or in-vitro methods for pathogenicity evaluation (Levisohn et al., 1986). Embryo mortality due to virulent MG was presented in eggs containing maternal MG antibody, although MG could be isolated from yolk sac membrane of live embryonated eggs after 17 days of inoculation (Levisohn et al., 1985). As in the case with *Mycoplasma synoviae*, embryo inoculation may reveal some aspects of MG infection, but it cannot reliably predict pathogenicity of a particular strain of MG in chickens (Kleven, 1985). A less pathogenic strain of MG would show almost 100 % mortality of chick embryos due to massive replication of cells in the inoculated animals especially where the observation period is long. It would indicate that mortality pattern of chick embryos by less pathogenic or moderately pathogenic MG is largely influenced by immunological status of the growing chick embryo (Levisohn et al., 1985) as well as history of exposure of the flocks, from where the fertile eggs are obtained, to other pathogens (Power and Jordan, 1976). The validity result of the in ovo pathogenicity evaluation of MG has been questioned (Levisohn et al., 1985). However, in ovo method has its own importance as it would reflect upon reduced hatchability. The gross lesions observed in embryos inoculated with the reference strain, vaccine strain and field isolates of MG were dwarfing, head edema, slightly enlarged and pale liver and spleen, and curled toes. These gross lesions findings were also reported in some other studies (Bradbury and McCarthy, 1983; Wakenell et al., 1995). In comparison, based on severity of gross lesions, the reference strain seems to be more pathogenic than the field isolates. This supports the report that MGS6 is one of the highly pathogenic strains of MG (Jordan, 1979; Power and Jordan, 1976).

Histopathological lesions of embryos from the reference, vaccine and field groups revealed that there were mild to severe infiltration of neutrophils and lymphocytes in the organs. These histopathological lesions were also reported by other researchers (Hayes et al., 1996; Lockaby et al., 1999; Mahmood et al., 1991). In this study, histopathological lesions in SPF embryonated

eggs from reference and vaccine groups showed more severe lesions than field groups, based on the lesion scoring of the liver, CAM, gizzard, lungs, heart, spleen, trachea, yolk sac, kidney, and bursa. This indicates that the reference strain of MG is more pathogenic than field MG isolates in the SPF embryos. While reference and vaccine groups showed almost similar lesions toward field groups in the organs of commercial embryos based on the lesion scoring of the liver, CAM, gizzard, lungs, heart, spleen, trachea, yolk sac, kidney, and bursa. This indicates that the reference strain and vaccine strain of MG are almost similar in pathogenicity to field MG isolates in the commercial embryos.

Mycoplasma gallisepticum were successfully reisolated from air sac, trachea, yolk, and CAM of the embryos, incomplete hatched chicks from pipped embryos and dead-in- shell embryos in SPF and commercial groups except those having moderate to severe caseous airsac lesion. This finding indicates that MG could possibly cause systemic infections in embryos. The reisolation of MG from some internal organs of experimentally infected embryos were also reported by other researchers, however, liver and brain were not examined (Bradbury and McCarthy, 1983; Reis and Yamamoto, 1971). MG was able to be reisolated from all 5 sites of samplings (choanal cleft, air sac, trachea, yolk, and CAM) from chick embryos and chicks (normal or poor quality chicks) that had no caseous airsac or caseous air score 1. Trachea and airsac were the best sites for the chick embryos and chicks, for detection of MG in this study. All MG inoculated samples were positive for MG by PCR.

Ten organs that were taken for histopathological lesions namely liver, CAM, gizzard, lungs, heart, spleen, trachea, yolk sac, kidney, and bursa showed that MG from reference, vaccine and field groups were pathogenic to embryos. Lungs, trachea, spleen, liver, CAM, and yolk sac showed mild to severe lesions histopathologically, thus, they are suitable to be taken as samples for MG isolation.

In conclusion, there was a significant difference in the pathogenicity of MG in SPF embryos based on weight but no significant difference was recorded in the commercial embryos. The severities of histopathological lesions of all sources were similar. No significant difference were observed in the histopathological lesion scoring in the embryonated chicken eggs from MG inoculated group of SPF and commercial embryos, based on the lesion scoring of liver, CAM, gizzard, lungs, heart, spleen, trachea, yolk sac, kidney, and bursa. Lesions are scored from 0 (no

lesion) to 3 (severe lesion). Caseous air sac lesion score ranged from 0 to 1 for SPF embryos and 1 to 3 lesion score for commercial broiler embryos.

Acknowledgements

This work was conducted under the financial support of University Putra Malaysia and Ministry of Science, Technology and Innovation (MOSTI), project number 02- 01- 04- SF0370.

Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Table legends

Table 1: Percentage of hatchability in SPF embryos.

Table 2: Percentage of hatchability in commercial chicken embryos

Table 1: Percentage of hatchability in SPF embryos.

Group	Fully Hatched		Pipped embryo		Dead-In-Shell	
	No.	%	No.	%	No.	%
Control	7/7	100	-	-	-	-
Broth	8/8	100	-	-	-	-
MG S6	-	-	5/10	50	5/10	50
TS 11	1/10	10	9/10	90	-	-
I-18	1/10	10	4/10	40	5/10	50
I-44	4/10	40	5/10	50	1/10	10
H21-8T	-	-	3/10	30	7/10	70
H21-11T	-	-	5/10	50	5/10	50
H24-5C	-	-	4/10	40	6/10	60
H26-9C	1/10	10	2/10	20	7/10	70

Table 2: Percentage of hatchability in commercial chicken embryos.

Group	Fully Hatched		Pipped embryo		Dead-In-Shell	
	No.	%	No.	%	No.	%
Control	7/7	100	-	-	-	-
Broth	8/8	100	-	-	-	-
MG-S6	10/10	100	-	-	-	-
ts-11	4/10	40	6/10	60	-	-
I-18	3/10	30	7/10	70	-	-
I-44	10/10	100	-	-	-	-
H21-8T	1/10	10	9/10	90	-	-
H21-11T	3/10	30	7/10	70	-	-
H24-5C	3/10	30	7/10	70	-	-
H26-9C	4/10	40	6/10	60	-	-

Figure legends

Figure 1: Mean of SPF embryos weight at different sampling time.

Figure 2: Mean of commercial embryos weight at different sampling time.

Figure 3: The ELISA titer pattern in commercial breeder chickens under the killed vaccine programs.

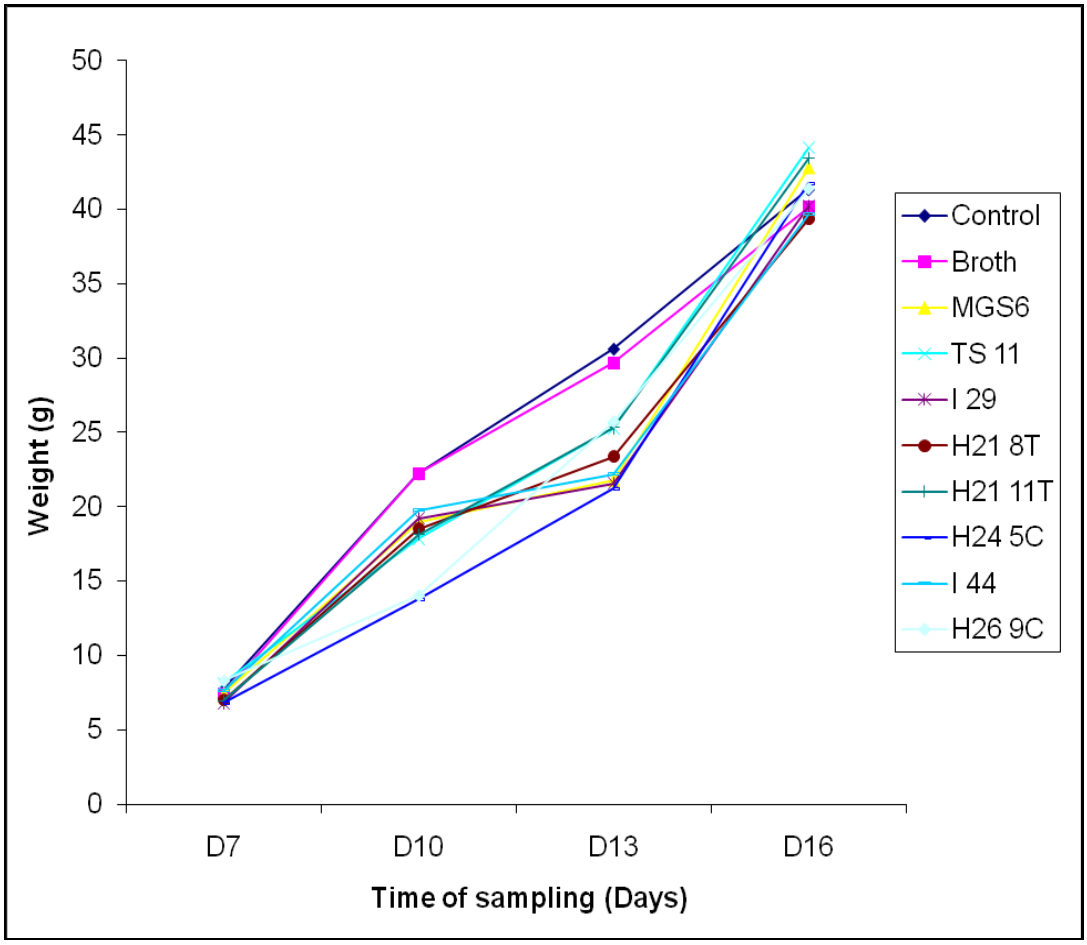

Figure 1: Mean of SPF embryos weight at different sampling time.

Mean of embryos weight from reference and field groups were lower ($p < 0.05$) than the mean of embryos weight from control group at day 10 and 13 pi. At day 7 and 15 pi, mean weight of control eggs is not significantly different ($p > 0.05$) from mean weight of MG reference group and mean weight of field group. At day 10 and 13 pi, mean weight of control eggs is significantly different from the mean weight of reference group and mean weight of field group.

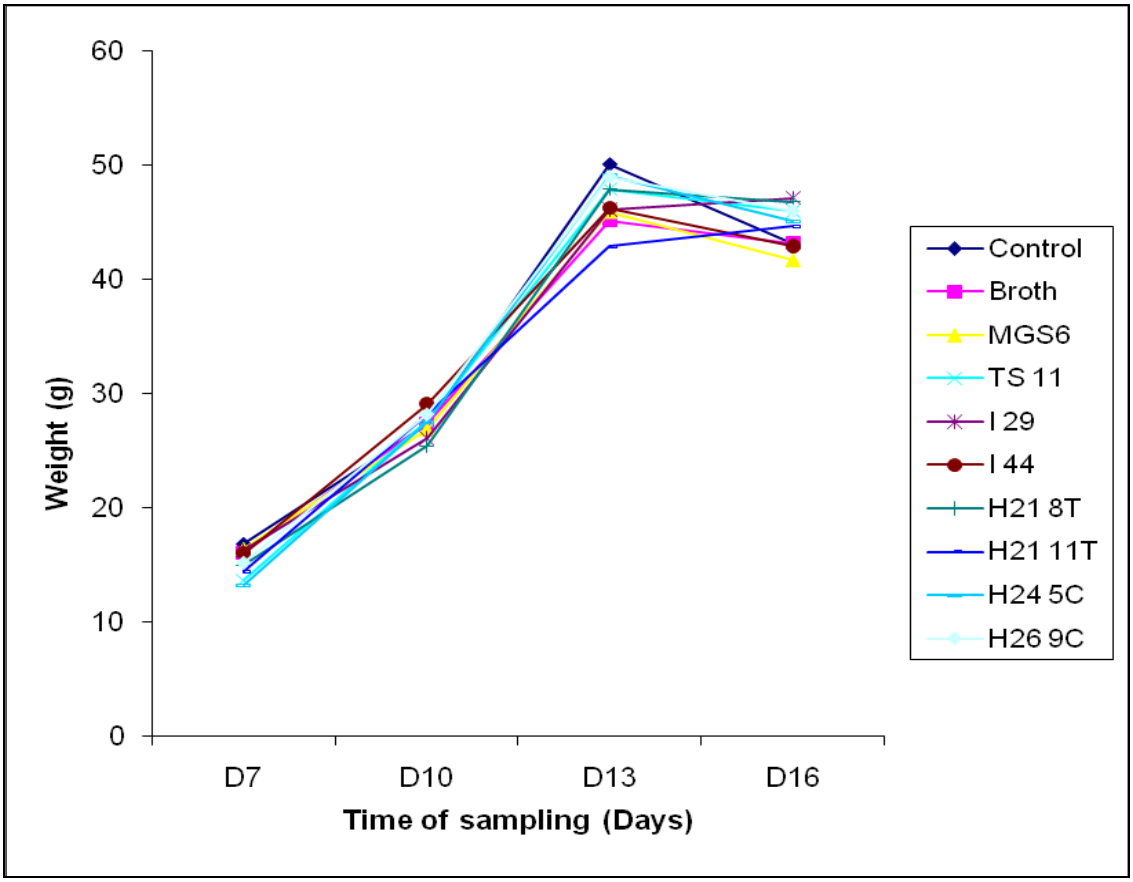

Figure 2: Mean of commercial embryos weight at different sampling time.

Mean of embryos weight from reference, vaccine and field groups were almost the same ($p > 0.05$) with the mean of embryos weight from control group for all the sampling. Mean weight of control eggs was not significantly different ($p > 0.05$) from mean weight of MG reference, vaccine and field groups for all time of samplings.

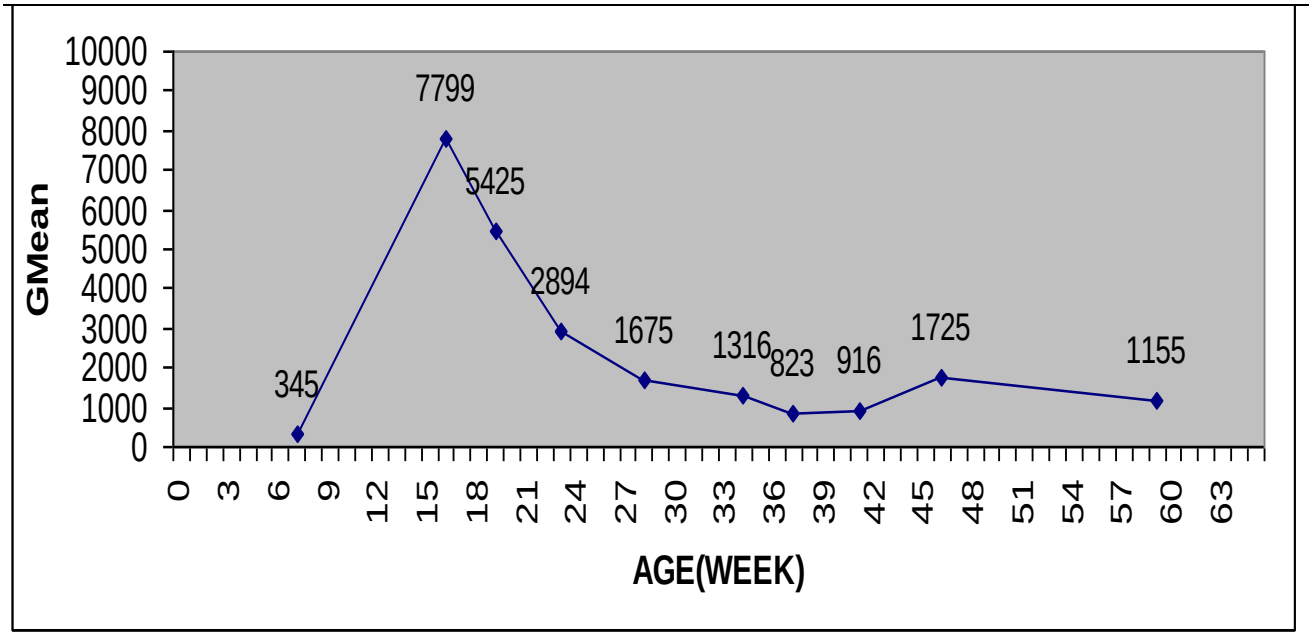

Figure 3: The ELISA titer pattern in commercial breeder chickens under the killed vaccine programs.

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