

The Efficacy of Tilmicosin against Broiler Chickens Infected with *Mycoplasma gallisepticum* Isolated in Thailand

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Abstract

Sixty 1-day-old-female broiler chickens were divided into groups as follows. Thirty birds were randomly selected to be bled from jugular vein for *Mycoplasma gallisepticum* (MG) antibody assay and swabbed at yolk sac for the detection of MG DNA by using PCR. The remaining birds were divided into three groups, 10 birds per group as follows. Group 1 was a sham negative control. Group 2 received MG serving as a positive control, whereas group 3 received MG and simultaneously treated with tilmicosin incorporated into drinking water. Each bird received MG that was injected into the left thoracic airsac, with 0.1 ml of inoculum containing MG organisms 10^6 CFU. Each group was raised on a wired cage in 3 isolated rooms with similar environmental conditions. Clinical sign and mortality rate were observed during 1-39 days old. Dead birds were necropsied and swabbed from left thoracic airsac for the detection of MG DNA. At 39 days old, all birds were bled, then necropsied to determine the gross airsac and microscopic tracheal lesion scores and simultaneously swabbed at the left thoracic air sac for the detection of MG DNA. Results revealed that the numbers of birds that showed clinical signs, mean gross airsac and microscopic tracheal lesion scores of group 3 were significantly less than those of group 2 ($p<0.05$).

Keywords : broiler chickens, *Mycoplasma gallisepticum*, Tilmicosin

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บทคัดย่อ

ประสิทธิภาพของยาทิลมิโคซินต่อไก่กระทงที่ได้รับเชื้อ มัยโคพลาสมา กัลลิเซปติกุม ที่แยกได้ในประเทศไทย

สมศักดิ์ ภัคภิณูโย* วิสุทธิ์ ระวีเวช วรฐ บุณศิริ ศุภสกล จรัสไพฑูรย์

ทำการเลี้ยงไก่กระทงเพศเมียอายุ 1 วัน จำนวน 60 ตัว สุ่มไก่จำนวน 30 ตัว เจาะเลือดที่เส้นเลือดดำที่คอเพื่อหาแอนติบอดีต่อเชื้อ มัยโคพลาสมา กัลลิเซปติกุม (เอ็มจี) และป้ายเชื้อจากถุงไข่แดงที่ผ่าซากเพื่อตรวจหาสารพันธุกรรมของเชื้อเอ็มจีโดยวิธี PCR แบ่งไก่ที่เหลือออกเป็น 3 กลุ่มกลุ่มละ 10 ตัว โดยกลุ่มที่ 1 เป็นกลุ่มควบคุมลบที่ไม่ได้รับการฉีดเชื้อเอ็มจีเข้าสู่ถุงลมทางด้านซ้ายของไก่ กลุ่มที่ 2 เป็นกลุ่มควบคุมบวกรับการฉีดเชื้อเอ็มจีเข้าสู่ถุงลมทางด้านซ้าย 0.1 มล. (10^6 CFU) ของไก่ และกลุ่มที่ 3 เป็นกลุ่มทดลองรับการฉีดเชื้อเอ็มจีเข้าสู่ถุงลมทางด้านซ้าย 0.1 มล. (10^6 CFU) ของไก่และมีการให้ยาทิลมิโคซินละลายในน้ำให้กิน จากนั้นนำไก่แต่ละกลุ่มแยกเลี้ยงในห้องปลอดเชื้อแต่ละห้องที่มีสิ่งแวดล้อมต่างๆคล้ายคลึงกัน ตลอดช่วงอายุตั้งแต่ 1-39 วัน เมื่ออายุครบ 39 วันจึงทำการเจาะเลือด ผ่าซากดูรอยโรคที่ถุงลมและท่อลม สังเกตอัตราการป่วย และอัตราการตาย ทำการป้ายเชื้อที่ถุงลมช่องอกด้านซ้ายเพื่อตรวจหาสารพันธุกรรมของเชื้อเอ็มจี พบว่าการป่วย รอยโรคทางจุลพยาธิวิทยาของท่อลม และรอยโรคทางมหพยาธิวิทยาของถุงลมในไก่กลุ่มที่ 3 น้อยกว่าในไก่กลุ่มที่ 2 อย่างมีนัยสำคัญทางสถิติ ($p < 0.05$)

คำสำคัญ : ไก่อกระทง มัยโคพลาสมา กัลลิเซปติกุม ยาทิลมิโคซิน

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Introduction

Mycoplasma gallisepticum (MG) infection is known as a chronic respiratory disease (CRD) in avian species (Kleven, 1998; Ley, 2003). Chickens, turkeys, quails, parrots, pheasants, pigeons, and peacocks are the natural hosts of MG infection (reviewed by Ley, 2003). 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. MG organisms of infected birds can be transmitted to the other birds via direct contact; that is, horizontal transmission. In addition, affected breeders can spread MG organisms through their progeny which is called as “vertical transmission” (Ley, 2003).

The economic losses due to 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, 2003).

For prevention, two types of vaccines; live and inactivated have been widely used in many countries (Ley, 2003) including Thailand. Strains of 6/85, ts-11, and F are commercially available, live, MG vaccines. Because each strain has a different degree of virulence, the use of live vaccines should be carefully considered and followed the manufacturers' instructions (Kleven, 1998, Ley, 2003). Inactivated MG vaccine is safer but needs 2 doses for optimal protection, plus the cost of individual bird injection and a reduced ability to control long-term infection in multiple age production units (Ley, 2003).

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. However, the gold standard for MG diagnosis is MG culture and isolation but this diagnosis requires MG antibody and a fluorescent microscope to determine the immunofluorescence technique (Ley, 2003). MG serology, such as serum plate agglutination (SPA), enzyme linked immunosorbent assay (ELISA) and hemagglutination inhibition (HI) tests are widely used in the laboratory; however, each method is limited by sensitivity and specificity (Kleven, 1998). SPA is used as the screening test because of its rapid, high sensitivity, low specificity, as well as being inexpensive. ELISA has been proved to have good sensitivity and more specificity compared to SPA procedure (Kleven, 1998). HI has high specificity but low sensitivity and no commercially available (Kleven, 1998). MG PCR is the rapid, high sensitivity and specificity method (Kempf et al., 1993; Jordan and Pattison, 1996).

Tilmicosin is a semi-synthetic antibiotic classified in group of macrolides and was approved in such countries for veterinary use in respiratory diseases of cattle and swine (Moore, 1996; Moore et al., 1996, Charleston et al., 1998). *In vitro* study showed that tilmicosin had an activity against Gram negative bacteria associated with respiratory disease including mycoplasmas (Ose, 1987; Jordan and Horrocks, 1996). Shryock et al. (1994) and Charleston et al. (1998) revealed that is effective to treat and control of MG inoculated broilers by tilmicosin administered in the diet and drinking water. For the pharmacokinetic of tilmicosin in chickens, Warren et al. (1997) showed that tilmicosin is distributed into lungs and airsacs in a dose of 2.30 ± 0.72 mg/g of lung following 48 hours of oral administration compared to the minimum inhibitory concentration (MIC) of MG determined by Ose in 1987 (0.048 µg/ml) and Jordan and Horrocks in 1996 (0.0125 mg/ml). In Thailand, Pakpinyo and Sasipreeyajan (2007) reported that the MIC of local MG isolates against tilmicosin ranged 0.099-12.5 mg/ml.

Tilmicosin can be incorporated into feed at 300-500 mg/kg (Shryock et al, 1994) or into water at 50-100 mg/l for either 3 or 5 days (Charleston et al.,

1998). Generally, the treatment of poultry diseases in the field usually incorporates into drinking water. Pakpinyo (2005) described that Thai's MG isolated could cause more severity of the respiratory signs, gross airsac and histopathologic lesion scores, morbidity and mortality compared to the MG reference strain (S6). Due to lack of data of the use of tilmicosin against the Thai's MG isolated; therefore, this study determined the efficacy of tilmicosin against broiler chickens infected with MG isolated in Thailand. The results of this study may be useful for Thai's poultry industry. The objective of this study was to determine the efficacy of tilmicosin incorporation in drinking water against broiler chickens infected with MG isolated in Thailand.

Materials and Methods

Sixty 1-day-old female broiler chickens were obtained from commercial breeder farm vaccinated with live MG vaccine at 6 weeks old. Thirty birds were randomized to bleed at jugular vein for MG serology, necropsied then swabbed at yolk sac for MG DNA by PCR procedure. Three cotton swabs were pooled as one sample, totally 10 samples for MG identification. Remaining birds were divided into 3 groups, 10 birds in each group. Each group was raised on a wired cage in 3 isolated rooms with the similar environmental condition and determined as follows.

Group 1: sham negative control. Birds were individually injected with 0.1 ml of FMS broth media at the left side of the middle line of the proximal end of the humerus and the pelvic girdle into the left thoracic airsac.

Group 2: positive control. Birds were individually injected 0.1 ml of inocula containing MG organisms 1.0×10^6 CFU into the left thoracic airsac as indicated in group 1.

Group 3: treatment. Birds were injected the same as group 2 and simultaneously treated with tilmicosin incorporated into drinking water at 75 mg/l/day for 3 consecutive days.

Feed and water were given *ad libitum*. All birds were observed for morbidity and mortality rate twice a day. The dead bird was necropsied to observe the airsac lesion, and simultaneously swabbed the left thoracic airsac to culture *E. coli* and to detect MG DNA by PCR procedure. At 39 days old, all remaining birds were bled for serology and identified the number prior to necropsy. The thoracic airsacs were grossly examined for typical of *Mycoplasma* infection, then swabbed for MG DNA by PCR procedure and *E. coli* isolation. Five tracheas from each group were submitted for histopathology. Each trachea was cross-sectioned into 4 pieces (1 proximal, 2 middle, 1 distal part of trachea). Lesion scores of airsacs and tracheas were blindly investigated.

Airsac lesion score. The airsac lesion score was grossly evaluated as 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 as 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. The maximum tracheal score for each bird was 12.

This experiment was approved by the Authors' Institution's Ethic Committee, and care was taken to minimize the number of animals used, issued by the Faculty of Veterinary Science, Chulalongkorn University, Number 44/2549, effective date: 13 September 2549-13

September 2550 (A.D.).

MG serology.

Serum plate agglutination (SPA): Fresh sera were tested against MG antigen (Nobilis®, Intervet International B.V., Holland), following the manufacturer's instructions. Briefly, thirty µl of serum were mixed with thirty µl of antigen and then incubated at room temperature for 1-2 min before the result was read. Negative and positive sera were included in each test.

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

MG DNA by PCR procedure: The broth samples were investigated in this study. This method was described by Lauerman (1998). The broth was extracted for the DNA template by centrifugation at 15,000xg, washing with distilled water, following by diluting the pellet with distilled water, boiling for 10 min, storing at -20°C for 10 min, centrifugation, and collecting the supernatant at -20°C until use. The PCR mixture, in a 50 µl volume, contained KCl 500 mM, Tris-HCl (pH 8.3) 100 mM, dNTP (Fermentas) 1 mM, primer F (5'GAGCTAAT CTGTAAAGTTGGTC3') and primer R (5'GCTTCCTT GCGGTTAGCAAC3') (Qiagen) 10 pmole each, Taq polymerase (Fermentas) 1.25 U and the DNA template, 5 µl (250 ng). The 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 over 40 cycles, followed by maintaining 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 an UV transilluminator and photographed.

Statistical analysis: Morbidity and mortality were analyzed statistically using a nonparametric Kruskal-Wallis test and Mann Whitney U test. The gross airsac and histopathologic tracheal lesion scores were examined by using Fisher's exact test. Statistical significance was determined as $p < 0.05$.

Results

The affected birds showed the respiratory signs including coughing, sneaking, watery ocular discharge and nasal discharge. The morbidity of group 1 (sham negative control), 2 (positive control) and 3 (treatment) ranged 0-6 birds. Numbers of sick birds of group 2 were significantly higher than those of groups 3 and 1, respectively. There was one dead bird observed only in group 3 (Table 1).

The numbers of birds aged 1 day old found positive reactors against SPA and ELISA was 0 and 3, respectively. At 39 days old, numbers of positive reactors of groups 1, 2 and 3 detected by SPA, and ELISA was 10, 10 and 9, and 0, 6 and 3, respectively (Table 2).

The mean gross airsac and histopathologic tracheal lesion scores of groups 2 were significantly higher than those of groups 3 and 1, respectively. Group 1 showed the lowest mean airsac and tracheal lesion scores and significant difference was observed compared to group 3 (Table 3).

The MG DNA detected by PCR procedure was not found in birds aged 1 day old. At 39 days old, only groups 2 and 3 detected MG DNA for 7 and 5 birds, respectively (Table 4). For one dead bird of group 3, MG DNA was not detected. *E. coli* could not be cultured from airsac swabs.

E. coli could not be isolated in all necropsied birds.

Discussion

This study revealed that the broiler chickens inoculated with the local MG isolated and simultaneously treated with tilmicosin (group 3) and the sham negative birds (group 1) had lower morbidity, gross airsac and histopathologic tracheal lesion scores compared to the birds inoculated with the local MG isolated (positive control; group 2). However, one dead bird was found in the treatment group. This dead bird possibly resulted from itself or management, not due to the virulence of MG infection, because none of any gross lesions, no growth of *E. coli* and no MG DNA was observed from this bird.

The antibody detected by the SPA and ELISA of birds aged 1 day old was found 3 out of 30 birds by the ELISA suggesting maternal derived antibody. In addition, no MG DNA was detected at 1 day old prior to separate birds by PCR procedure. The previous reasons indicated that 1 day old birds were free of the MG organisms or no vertical transmission; furthermore, at the 39 days old of birds of group 1 were still absent of MG antibody and organisms. There was a study described that passive transfer of high-titer antibodies could not protect MG infection (McMartin and Adler, 1961). Lam and Lin (1984), Talkington and Kleven (1985) and Whithear et al. (1990) revealed that the antibody titer against MG infection found in the blood did not correlate with the resistance of MG challenge. Therefore, the maternal derived antibody against MG failed to demonstrate any MG protective effect. The MG inoculated groups were found the positive reactors against MG organisms by the SPA and ELISA. From this study, group 2 showed the higher numbers of the positive reactors by the SPA compared with the ELISA. The SPA and ELISA can be used as the diagnosis method of serology, but the sensitivity and specificity of these tests were different. The SPA is much more sensitivity, whereas is lower specificity compared with the ELISA (Kleven, 1998). Furthermore, the SPA detects

Table 1 Morbidity and mortality during 1-39 days old period (n=10)

| Group | Morbidity | Mortality |
|-------|----------------|----------------|
| 1 | 0 ^a | 0 ^a |
| 2 | 6 ^b | 0 ^a |
| 3 | 3 ^c | 1 ^a |

^{a,b,c} the different superscript in the same column means significant different ($p<0.05$)

Table 2 Numbers of positive samples tested by SPA and ELISA of birds aged 1 and 39 days old

| Group | SPA | | ELISA | |
|-------|------------|-------------|------------|-------------|
| | 1 day old* | 39 days old | 1 day old* | 39 days old |
| 1 | | 0/10 | | 0/10 |
| 2 | 0/30** | 10/10 | 3/30 | 6/10 |
| 3 | | 2/9 | | 3/9 |

*Prior to separate birds

**Numbers of positive samples/total samples

Table 3 Mean of gross airsac lesion scores and mean of histopathologic tracheal lesion scores of birds aged 39 days old (Mean \pm SEM)

| Group | Gross airsac lesion scores | Histopathologic tracheal lesion scores |
|-------|-------------------------------------|--|
| 1 | 0.40 \pm 0.10 ^a (n=10) | 0.79 \pm 0.92 ^{a,b} (n=10) |
| 2 | 2.45 \pm 0.14 ^b (n=10) | 11.50 \pm 0.28 ^b (n=10) |
| 3 | 1.83 \pm 0.13 ^a (n=9) | 7.45 \pm 0.70 ^c (n=9) |

^{a,b} the different superscript in the same column means significant different ($p<0.05$)

Table 4 Numbers of positive samples detected by PCR of birds aged 1 and 35 days old.

| Group | PCR | |
|-------|------------|-------------|
| | 1 day old* | 39 days old |
| 1 | | 0/10 |
| 2 | 0/10** | 7/10 |
| 3 | | 5/10 |

*Prior to separate birds

**Numbers of positive samples/Total samples

IgM (immunoglobulin M), which is the first immunoglobulin produced from the immune response, presented at the first few days until 77 days of post infection (Kleven, 1975; Kleven, 1998). The ELISA detects IgG or IgA, which released after 14 days of post infection. This result also showed that the numbers of positive reactors detected by both SPA and ELISA of group 2 were less than those of

group 3. This finding was in accordance with Jordan et al. (1999) described that the infected and medicated poult had fewer numbers of the positive reactors than the infected and unmedicated poult.

The airsac and tracheal lesion scores of group 3 was higher than those of group 1 but was significant lower than group 2 suggesting that tilmicosin possibly reduced

the lesion scores of the inoculated birds. The result of the present study was similar to Charleston et al. (1998) revealed that the morbidity, the mean lesion score and the proportion of birds with severe lesions decreased after receiving tilmicosin as a treatment of MG infections in chickens. The mean peak plasma concentration of tilmicosin after administration in a single oral dose of 30 mg/kg was 2.09 ± 0.37 $\mu\text{g/ml}$ that is higher than the minimum inhibitory concentrations (MICs) for MG (0.0125-0.1 $\mu\text{g/ml}$) (Abu-Basha et al., 2007). The advantages of tilmicosin are that tilmicosin can be rapidly absorbed then distribute into tissues including lungs and airsacs as well as phagocyte cells including macrophages, monocytes and heterophils (Warren et al., 1997; Scoreneaux et al., 1998; Abu-Basha et al., 2007). Furthermore, the elimination of tilmicosin from the serum and lung was somewhat slow with mean half-lives of 30.18 and 75.74 hours, respectively (Abu-Basha et al., 2007). From rapid absorption, distribution, slow elimination and high plasma concentration, tilmicosin has potential to inhibit or eliminate MG organisms resulting in a small proportion of birds with severe lesions in the group 3.

In conclusion, tilmicosin incorporated in drinking water at a dose of 75 ppm for 3 consecutive days reduced the morbidity rate and the severity of gross airsac and histopathologic tracheal lesion scores in broilers inoculated with MG organisms at 1 day old.

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