Original Article

Antimicrobial susceptibility profiles (MIC and MMC) against *Mycoplasma gallisepticum* and *M. synoviae* isolated from chicken farms in Thailand

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Abstract

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) are important pathogens causing significant economic losses in poultry production. Disease prevention and control strategies include vaccination, antimicrobial therapy, and strict biosecurity measures. The present study provides, for the first time, both minimum inhibitory concentration (MIC) and minimum mycoplasmacidal concentration (MMC) values as antimicrobial susceptibility profiles of Thai MG and MS field isolates. This study aimed to evaluate the susceptibility of five MG and ten MS isolates to various antimycoplasma drugs: doxycycline, lincomycin-spectinomycin, tiamulin, tilmicosin, tylosin, and tylvalosin, using a microdilution broth assay. For MG isolates, tilmicosin had the highest mean MIC (12.5 µg/mL), while tylvalosin presented the lowest MIC (0.0488 µg/mL). For MS isolates, lincomycin-spectinomycin showed the highest MIC (0.2148 µg/mL), and tylvalosin performed the lowest (0.0488 µg/mL). This study also demonstrates the MMC values, showing tilmicosin had the highest MMC (12.5 µg/mL) for MG field isolates, while doxycycline had the highest MMC (0.3320 µg/mL) for MS field isolates. Tylvalosin consistently demonstrated the lowest MIC and MMC values for both MG and MS field isolates, suggesting its outstanding efficacy. Importantly, MMC values were introduced as a practical and insightful complement to MIC values by revealing viability of MG and MS field isolates at MIC levels of doxycycline (12 of 15 isolates), lincomycin-spectinomycin (7 of 15 isolates), tiamulin (1 of 15 isolates), tilmicosin (3 of 15 isolates), tylosin (4 of 15 isolates), and tylvalosin (2 of 15 isolates). These findings provide valuable insights for integrating MIC and MMC analysis into routine antimicrobial selection, contributing to more effective disease control, offering both growth inhibition and bactericidal effectiveness, and rational antimicrobial use in poultry farming, which could mitigate antimicrobial resistance, especially in poultry farms with intensive usage of antimicrobials.

Keywords: antimicrobial susceptibility profiles, chickens, minimum inhibitory concentration, minimum mycoplasmacidal concentration, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*

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Introduction

Mycoplasma gallisepticum (MG) infection has been known as a chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys (Armour, 2020), while *M. synoviae* (MS) infection is frequently found as a subclinical upper respiratory infection (Ferguson-Noel and Noormohammadi, 2020). MG infection can cause respiratory rales, coughing, nasal and ocular discharge, conjunctivitis, and even death in chickens (Pakpinyo and Sasipreeyajan, 2007), whereas the MS infection can perform systemic infection, causing lameness, infectious synovitis, tenovaginitis, bursitis, carcasses' condemnation, and reduced egg quality (Ferguson-Noel and Noormohammadi, 2020; Limpavithayakul *et al*., 2023). Due to the ability to transmit via horizontal and/or vertical routes and the high cost of disease-free flock maintenance, so other effective disease control strategies; proper farm management, biosecurity, and strategic medication; are suggested as alternative measure to reduce economic impact on poultry farms, reduce clinical signs, and improve production performances (Stipkovits and Kempf, 1996; Fiorentin *et al*., 2003; Kleven and Ferguson-Noel, 2008; Landman, 2008; Ley, 2008; Feberwee *et al*., 2009; Hong *et al*., 2015; Kreizinger *et al*., 2017; Armour, 2020; Ferguson-Noel and Noormohammadi, 2020; Limpavithayakul *et al*., 2023).

In several countries, medication has been a more common practice than using vaccines; however, excessive antimicrobial usage could predispose to the increasing antimicrobial resistance issues (Reinhardt *et al*., 2002; Wu *et al*., 2005; Pakpinyo and Sasipreeyajan, 2007; Li *et al*., 2010; Gerchman *et al*., 2011; Xia *et al*., 2015). Medication in infected flocks; using anti-mycoplasma drugs including tetracyclines, macrolides, lincosamides, quinolones, and pleuromutilins; could reduce antimicrobial use in the next and the progeny flocks, but the long intensive treatment is not generally acceptable (Fiorentin *et al*., 2003; Pakpinyo and Sasipreeyajan, 2007; Landman *et al*., 2008; Catania *et al*., 2010; Hong *et al*., 2015; Kreizinger *et al*., 2017). Furthermore, the antimicrobial resistance concern has influenced the practical use of antimicrobials for controlling MG or MS infection in poultry flocks, including the requirement of prescription from poultry veterinarians and the reliable susceptible profiles, including minimum inhibitory concentration (MIC) against MG or MS field isolates (Pakpinyo and Sasipreeyajan, 2007; Behbahan *et al*., 2008; Limpavithayakul *et al*., 2023). Presently, the antimicrobial susceptible profiles, including minimum mycoplasmacidal concentration (MMC) against MG or MS organisms by the microdilution broth method, have never been reported. The MMC is described as the lowest concentration of antimicrobial that stops growth after subculture onto or into a medium without antimicrobials; in practice, MMC represents the lowest antimicrobial concentration required to inactivate approximately 99.99% of mycoplasma organisms (Hannan, 2000). Therefore, in addition to first revealing the MMC values of various antimicrobials against MG and MS field isolates, this study aimed to determine the antimicrobial susceptibility profiles, including MIC and MMC, against MG and MS field isolates by the microdilution broth method.

Materials and Methods

Culture method: Frey mycoplasma broth base (HiMedia Laboratories Pvt. Ltd, Mumbai, India) was used in this study as previously described (Kleven, 1998). The sterile broth supplemented with 15% swine serum, dextrose, cysteine, nicotinamide adenine dinucleotide, penicillin, thallium acetate, and phenol red is referred to as Frey's broth medium supplemented with swine serum (FMS) (Kleven, 1998). All MG and MS strains from -80 °C were thawed at room temperature, and the broth cultures were subsequently re-cultured in fresh FMS broth at 37 °C in a humidified incubator for 3-5 days until the broth color changed from pink to red and to orange to yellow. Each cultured broth sample was then divided into two parts: the first part was enumerated as the color-changing unit (CCU) per ml, and the remaining part was immediately stored at -80 °C until further determining MICs and MMCs.

MG and MS field strains: All MG and MS isolates were obtained from commercial poultry farms, including broiler, layer, and breeder farms; in central and eastern parts of Thailand, swabbed by the farms' veterinarians and then submitted to the Avian Health Research Unit, Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, Thailand. Five MG and Ten MS field strains were stored at -80ºC since 2010 and 2019-2020, respectively. MG strains were confirmed by MG-specific PCR assays based on the 16sRNA gene (Lauerman, 1998) and the partial mgc2 gene (Ferguson *et al*., 2005), and MS strains were confirmed by MS-specific PCR assays based on the 16sRNA gene (Lauerman, 1998) and the partial vlhA gene (Wetzel *et al*., 2010). All MG and MS strains, as previously described, were obtained and used in the previous study (Pakpinyo and Sasipreeyajan, 2007; Limsatanun *et al*., 2022; Limpavithayakul *et al*., 2023). After being re-cultured and re-propagated in the FMS broth until color changing, each cultured broth was immediately divided into two parts: the first part for enumeration, and the remaining part for storing at -80 °C as the stock of inocula. The amount of mycoplasma organisms was prepared following standard protocols, with approximately 5×10^4 CCU/mL, aligning with established guidelines, which also state that the mycoplasma inoculum in MIC determination could be done in liquid or solid medium in the range of 10^3 to 10^5 CCU/mL or CFU per plate (Hannan, 2000). The use of experimental animals was approved by Chulalongkorn University Animal Care and Use Committee (IACUC), protocol No.1931051. The Biosafety and risk assessment was approved by the Institution Biosafety Committee (CU-VET-BC), protocol No.2331002.

Antimicrobials: Six antimicrobials used in this study were registered and approved by the Food and Drug Administration, Ministry of Public Health, Thailand. Antimicrobials, including doxycycline (SOLUDOX 50%, Synbiss Co., Ltd), lincomycin-spectinomycin

(Linco-Spectin™ 100, Zoetis [Thailand] Ltd.), tiamulin (Denagard™ 45%, Elanco [Thailand] Ltd.), tilmicosin (Pulmotil™ AC, Elanco [Thailand] Ltd.), tylosin (Pharmasin WSG, Huvepharma [Thailand] Ltd.), and tylvalosin (Valosin® SOLUBLE, ECO Animal Health) were formulated and diluted in FMS broth.

Determination of MICs by a serial dilution broth methods: The determination of MICs by a serial broth dilution method was previously described (Wang *et al*., 2001; Pakpinyo and Sasipreeyajan, 2007). Briefly, duplicate wells of antimicrobials were twofold, serially diluted in 50 µl of FMS broth in sterile 96-well, flat-bottom microtitration plates. The 50 µL of FMS broth containing MG or MS organisms was added to each well containing various antimicrobials. The final concentrations of antimicrobials were 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.7813, 0.3906, 0.1953, 0.0977, and 0.0488 µg/mL. The final concentration of the MG or MS organisms was approximately 5×10^4 CCU/mL (Hannan, 2000). All plates were sealed with plastic sheets and incubated at 37 °C under a humidified condition. After incubation, the positive and negative controls, including only MG or MS organisms and FMS broth, respectively, were also added in the last two columns of each plate. The MICs were recorded 10 days after the positive control broth color was changed. The lowest concentration of each antimicrobial, which inhibited the broth change of color from red to yellow, was recorded as the MIC value. The MIC breakpoints of avian mycoplasmas against six tested antimicrobials were based on the previous MIC determination (Table 1) (Hannan, 2000; Landman *et al*., 2008; Kreizinger *et al*., 2017).

Determination of MMCs by a serial dilution broth method: The determination of MMCs by a serial broth microdilution method was modified from Hannan (2000). Briefly, at the well of the MIC and wells of higher concentration of antimicrobial prior to the MIC, 100 µL of each incubated broth of these wells was sub-cultured by diluting into each 100 µL of fresh FMS broth without antimicrobial in the 96-well, flat-bottom microtitration plates. The positive and negative controls of MG or MS and only FMS broth, respectively, were included in each test. All plates were sealed with plastic sheets and incubated at 37 °C in a humidified condition for 10 days. The lowest concentration of each antimicrobial that completely inhibited the growth upon subculture, or the broth change of color from red to yellow, was recorded as MMC values.

Table 1 MIC breakpoints of avian mycoplasmas against six tested antimicrobials.

Antimicrobials	Susceptible (µg/mL)	Resistant (µg/mL)
Doxycycline[a,b]	≤ 4	≥ 16
Lincomycin-spectinomycin[b]	≤ 2	≥ 8
Tiamulin[b]	≤ 8	≥ 16
Tilmicosin[a,b]	≤ 8	≥ 32
Tylosin[a,c]	≤ 1	≥ 4
Tylvalosin[b]	≤ 0.5	≥ 2

[a]MIC breakpoints according to Landman *et al*. (2008)

[b]MIC breakpoints according to Kreizinger *et al*. (2017)

[c]MIC breakpoints according to Hannan (2000)

Results

MIC and MMC values of tested antimicrobials, doxycycline, lincomycin-spectinomycin, tiamulin, tilmicosin, tylosin, and tylvalosin, against five MG, ten MS, and reference strains, MG S6 and MS WVU 1853, were shown in Tables 2, 3, 4, and 5. MIC values showed that for Thai MG isolates, tilmicosin and tylvalosin performed the highest mean MIC (12.5 µg/mL) and the lowest mean MIC (0.0488 µg/mL), respectively. Whereas, among Thai MS isolates, lincomycin-spectinomycin and tylvalosin exhibited the highest mean MIC (0.2148 µg/mL) and the lowest mean MIC (0.0488 µg/mL), respectively. Besides, MMC values indicated that for Thai MG isolates, tilmicosin showed the highest MMC (12.5 µg/mL), while tiamulin and tylvalosin presented the lowest MMC (0.0684 µg/mL). For Thai MS isolates, doxycycline and tylvalosin exhibited the highest MMC (0.3320 µg/mL) and the lowest (0.0488 µg/mL), respectively.

MG field strains were presented the MIC values against doxycycline, lincomycin-spectinomycin, tiamulin, tilmicosin, tylosin, and tylvalosin in ranges (mean ± SE) as 0.0488 – 0.0977 (0.0586 ± 0.0098), 0.7813 – 0.7813 (0.7813 ± 0.0), 0.0488 – 0.0977 (0.0684 ± 0.0120), 12.5 – 12.5 (12.5 ± 0.0), 0.1953 – 0.7813 (0.3906 ± 0.1070) and 0.0488 – 0.0488 (0.0488 ± 0.0) µg/mL, respectively. While the MMC values of MG field strains against doxycycline, lincomycin-spectinomycin, tiamulin, tilmicosin, tylosin, and tylvalosin were shown in ranges (mean ± SE) as 0.0488 – 0.0977 (0.0781 ± 0.012), 0.7813 – 3.1250 (1.5625 ± 0.0), 0.0488 – 0.0977 (0.0684 ± 0.012), 12.5 – 12.5 (12.5 ± 0.0), 0.3906 – 1.5625 (0.0731 ± 0.2278) and 0.0488 – 0.0977 (0.0684 ± 0012) µg/mL, respectively.

Besides, MS field strains were presented the MIC values against doxycycline, lincomycin-spectinomycin, tiamulin, tilmicosin, tylosin, and tylvalosin in ranges (mean ± SE) as 0.0488 – 0.3906 (0.1611 ± 0.0309), 0.0977 – 0.3906 (0.2148 ± 0.0319), 0.0488 – 0.1953 (0.0684 ± 0.0149), 0.0488 – 0.3906 (0.1709 ± 0.0484), 0.0488 – 0.1953 (0.0732 ± 0.0150), and 0.0488 – 0.0488 (0.0488 ± 0.0) µg/mL, respectively, and the MMC values of MS field strains against doxycycline, lincomycin-spectinomycin, tiamulin, tilmicosin, tylosin, and tylvalosin were shown in ranges (mean ± SE) as 0.0488 – 0.0977 (0.0781 ± 0.012), 0.7813 – 3.1250

(1.5625 ± 0), 0.0488 – 0.0977 (0.0684 ± 0.012), 12.5 – 12.5 (12.5 ± 0), 0.3906 – 1.5625 (0.0731 ± 0.2278) and 0.0488 – 0.0977 (0.0684 ± 0012) µg/mL, respectively.

In addition, MMC values of MG and MS field isolates were presented at a higher level than MIC values in doxycycline (12 of 15 isolates), lincomycin-spectinomycin (7 of 15 isolates), tiamulin (1 of 15 isolates), tilmicosin (3 of 15 isolates), tylosin (4 of 15 isolates), and tylvalosin (2 of 15 isolates).

Table 2 Details of MG strains and MIC values of MG strains.

Strain ID	MIC values (µg/ml)					
	DX	LC-SP*	TIA	TIL	TYL	TVN
AHRU2003CU5801	0.0488	0.7813	0.0488	12.5	0.7813	0.0488
AHRU2009CU2001	0.0488	0.7813	0.0488	12.5	0.3906	0.0488
AHRU2002CU0101	0.0977	0.7813	0.0977	12.5	0.3906	0.0488
AHRU2003CU0109	0.0488	0.7813	0.0488	12.5	0.1953	0.0488
AHRU2002CU3101	0.0488	0.7813	0.0977	12.5	0.1953	0.0488
S6	0.0488	1.1719	0.0488	0.0488	0.0488	0.0488
Mean ± SE**	0.0586 ± 0.0098	0.7813± 0.0000	0.0684 ± 0.0120	12.5 ± 0.0000	0.3906 ± 0.1070	0.0488± 0.0000

*Abbreviations of antibiotics: DX doxycycline, LC-SP lincomycin-spectinomycin, TIA tiamulin, TIL tilmicosin, TYL tylosin, TVN tylvalosin
**Excluding S6 strain

Table 3 Details of MG strains and MMC values of MG strains.

Strain ID	MMC values (µg/ml)					
	DX	LC-SP*	TIA	TIL	TYL	TVN
AHRU2003CU5801	0.0488	0.7813	0.0488	12.5	1.5625	0.0977
AHRU2009CU2001	0.0488	1.5625	0.0488	12.5	0.7813	0.0488
AHRU2002CU0101	0.0977	3.1250	0.0977	12.5	0.3906	0.0488
AHRU2003CU0109	0.0977	1.5625	0.0488	12.5	0.3906	0.0488
AHRU2002CU3101	0.0977	1.5625	0.0977	12.5	0.3906	0.0977
S6	0.0488	1.5625	0.0488	0.0488	0.0488	0.0488
Mean ± SE**	0.0781 ± 0.0120	1.5625 ± 0.0000	0.0684 ± 0.0120	12.5 ± 0.0000	0.7031 ± 0.2278	0.0684 ± 0.0120

*Abbreviations of antibiotics: DX doxycycline, LC-SP lincomycin-spectinomycin, TIA tiamulin, TIL tilmicosin, TYL tylosin, TVN tylvalosin
**Excluding S6 strain

Table 4 Details of MS strains and MIC values of MS strains.

Strain ID	MIC values (µg/ml)					
	DX	LC-SP*	TIA	TIL	TYL	TVN
AHRU2015CU2802	0.3906	0.1953	0.0977	0.0977	0.0977	0.0488
AHRU2020CK3021	0.0977	0.3906	0.0488	0.0977	0.0488	0.0488
AHRU2020CU1402	0.0977	0.1953	0.0488	0.0488	0.0488	0.0488
AHRU2020CU1409	0.1953	0.0977	0.0488	0.0488	0.0488	0.0488
AHRU2020CK0404	0.0977	0.1953	0.0488	0.3906	0.0977	0.0488
AHRU2020CK0301	0.1953	0.3906	0.1953	0.3906	0.1953	0.0488
AHRU2020CK0305	0.0977	0.1953	0.0488	0.3906	0.0488	0.0488
AHRU2020CK0705	0.1953	0.1953	0.0488	0.0977	0.0488	0.0488
AHRU2020CK0709	0.1953	0.0977	0.0488	0.0977	0.0488	0.0488
AHRU2020CU1323	0.0488	0.1953	0.0488	0.0488	0.0488	0.0488
WVU1853	0.0488	0.0977	0.0488	0.0488	0.0488	0.0488
Mean ± SE**	0.1611 ± 0.0309	0.2148 ± 0.0319	0.0684 ± 0.0149	0.1709 ± 0.0484	0.0732 ± 0.0150	0.0488 ± 0.0000

*Abbreviations of antibiotics: DX doxycycline, LC-SP lincomycin-spectinomycin, TIA tiamulin, TIL tilmicosin, TYL tylosin, TVN tylvalosin
** Excluding WVU1853 strain

Table 5 Details of MS strains and MMC values of MS isolates.

Strain ID	MMC values (µg/ml)					
	DX	LC-SP*	TIA	TIL	TYL	TVN
AHRU2015CU2802	0.7813	0.1953	0.0977	0.1953	0.0977	0.0488
AHRU2020CK3021	0.1953	0.3906	0.0488	0.1953	0.0488	0.0488
AHRU2020CU1402	0.1953	0.3906	0.0488	0.0488	0.0488	0.0488
AHRU2020CU1409	0.3906	0.0977	0.0488	0.0488	0.0488	0.0488
AHRU2020CK0404	0.1953	0.1953	0.0977	0.3906	0.0977	0.0488
AHRU2020CK0301	0.3906	0.7813	0.1953	0.7813	0.1953	0.0488
AHRU2020CK0305	0.1953	0.1953	0.0488	0.3906	0.0488	0.0488
AHRU2020CK0705	0.3906	0.1953	0.0488	0.0977	0.0488	0.0488
AHRU2020CK0709	0.3906	0.1953	0.0488	0.0977	0.0488	0.0488
AHRU2020CU1323	0.1953	0.1953	0.0488	0.0488	0.0488	0.0488
WVU1853	0.0488	0.0977	0.0488	0.0488	0.0488	0.0488
Mean ± SE**	0.3320 ± 0.0586	0.2832 ± 0.0625	0.0732 ± 0.0150	0.2295 ± 0.0739	0.0732 ± 0.0150	0.0488 ± 0.0000

*Abbreviations of antibiotics: DX doxycycline, LC-SP lincomycin-spectinomycin, TIA tiamulin, TIL tilmicosin, TYL tylosin, TVN tylvalosin

**Excluding WVU1853 strain

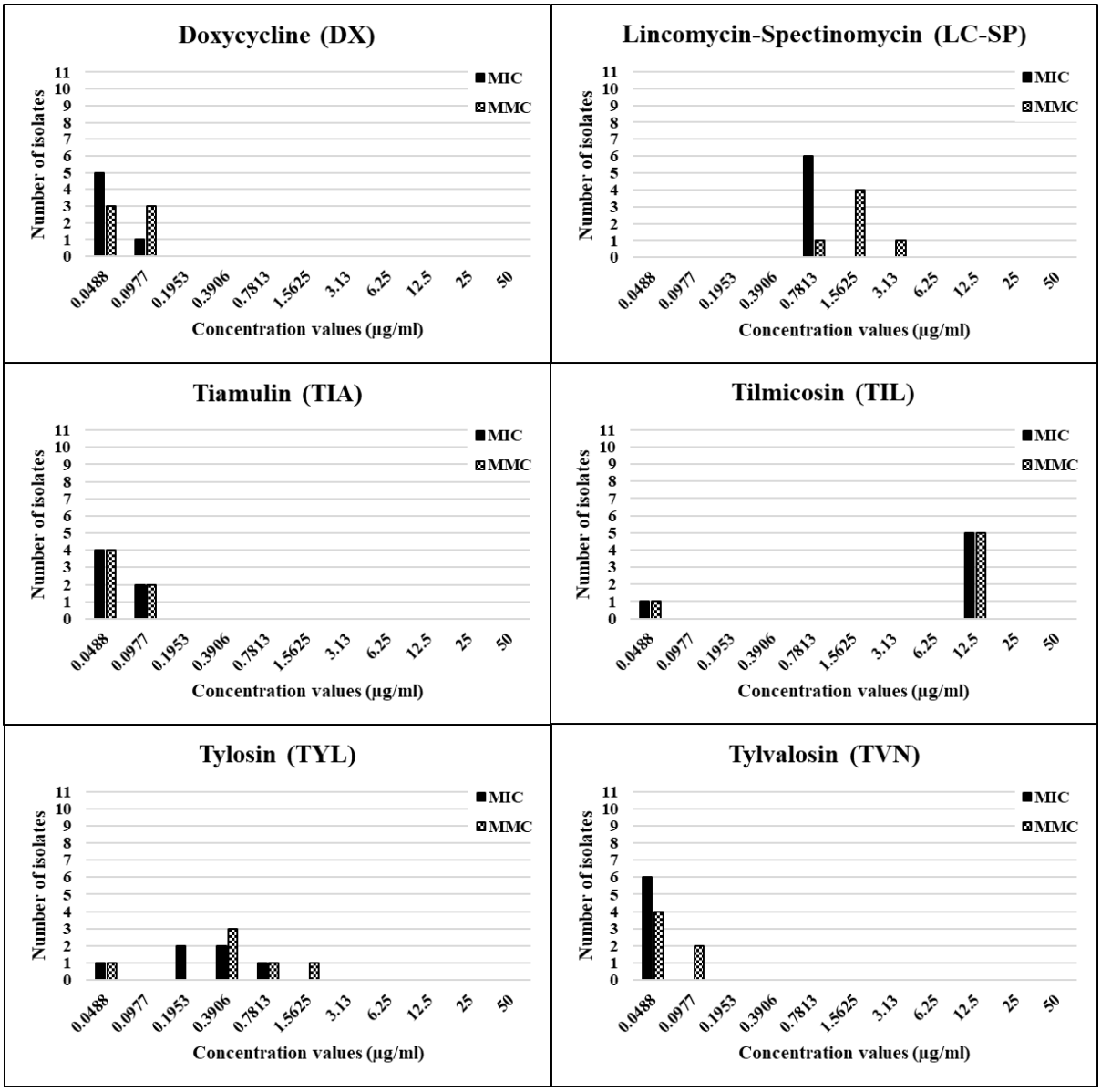

Figure 1 Bar charts present the distribution of MIC and MMC values of tested antimicrobials, including doxycycline, lincomycin-spectinomycin, tiamulin, tilmicosin, tylosin, and tylvalosin against MG strains.

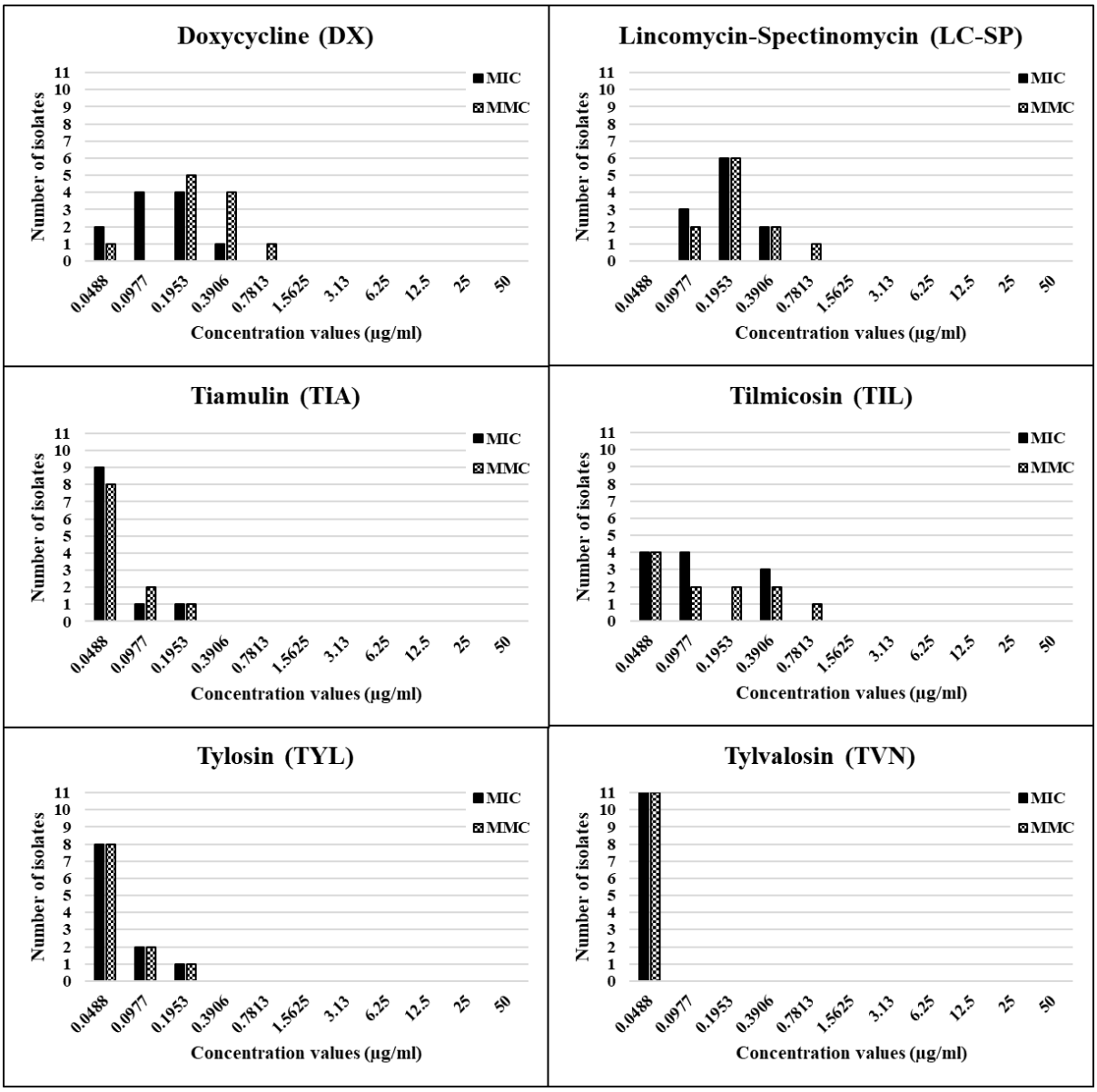

Figure 2 Bar charts present the distribution of MIC and MMC values of tested antimicrobials, including doxycycline, lincomycin-spectinomycin, tiamulin, tilmicosin, tylosin, and tylvalosin against MS strains.

Discussion

This study presents the first report of MMC values for Thai MG and MS field isolates, alongside corresponding MIC values. Despite the limited number of isolates, the MIC and MMC values obtained in this study provide useful information for the poultry industry, especially the poultry veterinarians responsible for deciding or selecting appropriate antimycoplasma drugs to control or treat the mycoplasma infections in poultry flocks. Rational antimicrobial usage in the poultry industry has become the current recommendation to avoid further resistance against antimycoplasma drugs, which are mostly predisposed by improper or excessive antimicrobial use. In this study, tylvalosin demonstrated the lowest MIC and MMC values among the tested drugs, suggesting its superior efficacy against MG and MS isolates, for which similar findings have been reported in previous studies (Cerda *et al*., 2002; Kreizinger *et al*., 2017; Abd El-Hamid *et al*., 2019; Morrow *et al*., 2020).

Several antimicrobials could be used to manage mycoplasma infections in poultry; for reducing the severity or for treating mycoplasma infection, for example, group tetracyclines (e.g., chlortetracycline, doxycycline, oxytetracycline), group lincosamides (e.g., lincomycin), group pleuromutilin (e.g., tiamulin), and group macrolides (e.g., tilmicosin, tylosin, tylvalosin), which are protein synthesis inhibitors of bacteria (Mund *et al*., 2017; Armour, 2020). So, in this study, antimicrobials including doxycycline, lincomycin-spectinomycin, tiamulin, tilmicosin, tylosin, and tylvalosin, which are registered and approved by the Food and Drug Administration, Ministry of Public Health, Thailand, were used to determine antimicrobial susceptible profiles; MIC and MMC; against MG and MS strains by microdilution broth assay which is the simplicity and convenience to determine small numbers of mycoplasma strains (Hannan, 2000).

The present study revealed that the MIC values of doxycycline against MG strains were lower than those of MS strains, which were similar to the MIC values investigated in European countries (El-Hamid *et al*., 2019; Jong *et al*., 2021). Although the accurate reason for this difference is not fully clarified, it may reflect intrinsic species-level differences in membrane permeability, target-site affinity, or efflux pump activity between MG and MS, which should be further investigated. Additionally, while tiamulin exhibited low MIC values against both MG and MS, its clinical use requires caution due to its known adverse interactions with ionophore anticoccidials such as monensin, narasin, and salinomycin, which can result in toxicity and increased mortality in poultry (Horrox, 1980). In addition to susceptibility data, the pharmacological and management issues should also be considered when selecting antimicrobials in the poultry industry.

For lincomycin-spectinomycin, only lincomycin affects mycoplasmas; the mean MIC value of the MG S6 strain was quite higher compared with other MG field strains. Surprisingly, the MG S6 strain used as a reference strain in this study has never been exposed to any antimycoplasma drugs and still exhibited the high MIC as previously published (Pakpinyo and Sasipreeyajan, 2007). In comparison with other tested drugs, the lincomycin-spectinomycin might not be suitable to determine the MIC against the MG S6 strain. Consistent with previous reports, this study also revealed the elevated MIC values for lincomycin-spectinomycin, suggesting limited efficacy against MG and MS strains and potential unsuitability as a first-line treatment (Pakpinyo and Sasipreeyajan, 2007; Limpavithayakul *et al*., 2023).

In addition, tilmicosin evaluation in this study exhibited the highest MIC values against MG field isolates, followed by lincomycin-spectinomycin, tylosin, tiamulin, doxycycline, and tylvalosin, respectively, while lincomycin-spectinomycin performed the highest MIC values again against MS field isolates, followed by tilmicosin, doxycycline, tylosin, tiamulin and tylvalosin, respectively. Although tilmicosin and tylvalosin, classified as macrolides, are semisynthetic derivatives of the natural product tylosin (EFSA Panel on Biological Hazards, 2021), however, tylvalosin could show an ability to enter and accumulate in cells, especially white blood cells, greater than either tilmicosin or tylosin (Stuart *et al*., 2007). Tilmicosin was reported to have the ability in the laboratory to be more rapidly stimulate resistant mutants of MG than tylosin (Wu *et al*., 2005). Even though the mean MIC value of tilmicosin was highest, the efficacy of tilmicosin against MG-infected chickens exhibited interesting results, including reducing morbidity and lesion severity of grossly airsac and microscopically tracheal lesion scores (Pakpinyo *et al*., 2008). Jong *et al*. (2021) described MIC90 values of tilmicosin against 65 MG isolates in Italy and Spain were 32, ranging from <0.001-64, which were quite similar to the MIC values of the present study.

Interestingly, tylvalosin, a recent addition to poultry industry in Thailand, showed the lowest MIC and MMC values, confirming its potential as an effective antimicrobial agent (Wang *et al*., 2001; Morrow *et al*., 2020). Several publications found that tylvalosin had the lowest MIC values compared with other tested drugs, which was concordant with this study (Wang *et al*., 2001; Cerda *et al*., 2002; Kreizinger *et al*., 2017; Abd El-Hamid *et al*., 2019; Morrow *et al*., 2020). Furthermore, there was an experimental study on tylvalosin and tiamulin at the same dose against MS growth in chicken embryos, resulting in tylvalosin showing a greater inhibitory effect on MS growth in chicken embryos than tiamulin (Wang *et al*., 2001).

Up to the present, any information available on MMC values against MG and MS strains has not been described. Therefore, this is the first report about MMC values against MG and MS isolates from Thailand. The MMC procedure, which was modified from Hannan (2000), provides valuable information on bactericidal activity and complements MIC testing in clinical settings. Overall, tylvalosin consistently showed the lowest mean MMC value, followed by tiamulin and tylosin, respectively, whereas the mean MMC value of doxycycline, lincomycin-spectinomycin, and tilmicosin were different. This highlights tylvalosin's potential for effective use in controlling *Mycoplasma* infections in poultry. Compared with the MIC value, the MMC value of tylvalosin, doxycycline, tylosin, and lincomycin-spectinomycin against MG strains and of tiamulin, tilmicosin, lincomycin-spectinomycin, and doxycycline against MS strains were orderly increased. In practice, MIC is the inhibitory mycoplasmas at the lowest concentration of antimicrobial, while MMC is the inactivated mycoplasmas at approximately 99.99 % at the lowest concentration of antimicrobial (Hannan, 2000). Although the MIC of the present study was incubated between antimycoplasma drugs and MG or MS isolates for 10 days, MG or MS of some drugs were still alive, which were further confirmed by the MMC assay. Importantly, despite the limited number of isolates in this study, significant differences in MIC and MMC values were observed. MMC values revealing viability of MG and MS field isolates at MIC levels of doxycycline (12 of 15 isolates), lincomycin-spectinomycin (7 of 15 isolates), tiamulin (1 of 15 isolates), tilmicosin (3 of 15 isolates), tylosin (4 of 15 isolates), and tylvalosin (2 of 15 isolates) demonstrate that MMC can disclose the critical clinical differences not detected by MIC alone. These findings provide valuable insights for integrating MIC and MMC analysis into routine antimicrobial selection, contributing to more effective control and rational antimicrobial use in poultry farming, which could mitigate antimicrobial resistance, especially in poultry farms with intensive usage of antimicrobials. MMC could be applied as a practical complement to MIC, with direct implications for clinical decision-making and antimicrobial usage in poultry production.

In this study, the tested MG and MS isolates were only third or fourth passage, so the number of microbial passages did not affect the resistant test, as previously evident, showing that microbial passages could lead to the selection of resistant mutants in the resistant test (Gautier-Bouchardon *et al*., 2002). However, the lack of molecular analysis of resistance genes in this study would have provided further insight into observed phenotypic resistance patterns

and detection of resistance-associated mutations or gene profiling to elucidate mechanisms underlying reduced susceptibility as previously studied in quinolone resistance-determining regions (QRDR) of DNA gyrase gene, gyrA and gyrB genes, and QRDR of topoisomerase IV, parC and parE genes (Le Carrou *et al*., 2006).

Besides, in addition to the further study about the history of antibiotic usage in the poultry farms including the effect of frequent or long period usage, the antibiotic resistance development also requires the further confirmatory methods; PCR, sequencing or biochemical assays; to encourage the molecular assays, although the MG and MS isolates were identified using 16S rRNA-based PCR assay and additional targets gene-based PCR assay (mgc2 and vlhA for MG and MS, respectively). Furthermore, although the reference strains of MG (S6 strain) and MS (WVU 1853) participated in this study presented further studies should be planned to determine the known resistant and sensitive strains as controls for benchmarking antimicrobial response.

In conclusion, MIC and MMC values obtained in this study contribute essential information for poultry veterinarians regarding antimicrobial selection, especially tylvalosin, demonstrating the superior antimicrobial efficacy against MG and MS isolates with the lowest MIC and MMC values among the tested drugs. Besides, this study is the first report of MMC values against MG and MS field isolates in Thailand. The MMC assay provides valuable information regarding bactericidal effects and may complement MIC testing in clinical decision-making. The findings highlight the integration of MIC and MMC analysis into routine antimicrobial selection, contributing to more effective disease treatment and rational antimicrobial use, mitigating resistance development in poultry production. This study determined MIC and MMC values for MG and MS isolates from Thai poultry farms. Tylvalosin exhibited the lowest MIC and MMC values, suggesting their high efficacy. The MMC assay represents a practical approach for evaluating antimicrobial effectiveness and could be further used in the conventional mycoplasma laboratory. These data will assist poultry veterinarians in selecting appropriate antimicrobials and preventing resistance development.

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