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Prevalence and antimicrobial susceptibility of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* isolated from the central peninsular Malaysia

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ABSTRACT

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) have been recognized and listed by the World Organization for Animal Health (WOAH) as the two most pathogenic avian mycoplasma species. These organisms can cause a wide range of symptoms in birds, including acute and chronic respiratory diseases, synovitis, air sacculitis, severe weight loss, eggshell abnormalities, and reduced egg production. In Malaysia, previous studies have mainly focused on seroprevalence, and prevalence based on polymerase chain reaction (PCR) with molecular characterization, which have confirmed the presence of MG and MS among the poultry population. These could pose significant economic challenges for farmers. To investigate this further, the present study was conducted to determine the prevalence and the antimicrobial minimum inhibitory concentrations (MIC) of MG and MS isolates that are circulating in the farms in central region of Peninsular Malaysia. A total of 407 choanal cleft swab samples were collected from various poultry farms in the central Peninsular and were subjected to isolation and PCR. Of these, 103 (25.3 %) MG, 173 (42.5 %) MS and 36 (8.8 %) MG-MS were obtained by PCR, while 61 (15.%) 14(3.4 %) and 1 (0.2 %) successful isolation of MG, MS, and MG-MS were made respectively. Antimicrobial susceptibility testing showed that MG and MS isolates had reduced susceptibility to lincomycin (4 - ≥ 32 μg/mL) and erythromycin (≤0.12 - ≥ 64 μg/mL) respectively, while Tylvalosin (≤0.015 – 0.5 μg/mL), Tiamulin (≤0.015 – 0.25 μg/mL) and Doxyccline (0.06 -0.5 μg/mL) showed the highest susceptibility for these organisms. These findings highlights that while the threat of MG and MS infections persist, special attention should be directed towards the growing prevalence of MS. Furthermore, regular monitoring of antimicrobial susceptibility profiles should guide treatment strategies and address potential concerns regarding antimicrobial resistance.

Introduction

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are the primary agents causing avian mycoplasmosis, leading to considerable economic impacts within the global poultry sector (Wu et al., 2024). MG is mainly linked to chronic respiratory disease in chickens, resulting in lower egg production, reduced weight gain, and increased mortality rates (Wu et al., 2022). Although MS typically presents as a subclinical upper respiratory tract infection, it can also progress to severe clinical signs and lesions, particularly when co-infections with other pathogens like Escherichia coli occur (Xu et al., 2020). More so, infectious synovitis, which is characterised by joint swelling and lameness due to inflammation of the synovial membranes, is known to be caused by MS in chickens and turkeys (Amer et al., 2019). One important consequence of MS is its correlation with eggshell apex abnormalities (EAA) in laying hens, which has been closely associated with the pathogen's oviduct colonization (Feberwee et al., 2009). In Southeast Asia, including Malaysia, disease control has largely relied on routine prophylactic use

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of antimicrobials. MS infection usually starts as a subclinical upper respiratory tract infection, it can also develop into serious clinical symptoms and lesions, especially if other pathogens, such as Escherichia coli, co-infect the host (Xu et al., 2020). Moreover, MS is known to cause infectious synovitis in chickens and turkeys, which is characterised by joint swelling and lameness brought on by inflammation of the synovial membrane (Amer et al., 2019). Another notable impact of MS is its association with eggshell apex abnormalities (EAA) of laying hens which has been closely linked to the colonization of the oviduct by the pathogen (Feberwee et al., 2009).

The conventional use of antibiotics has been the cornerstone of disease control efforts in Malaysia and by extension Southeast Asia. However, the effectiveness of this approach for the most part is undermined by the seeming dearth of information on antimicrobial susceptibility patterns and minimum inhibitory concentrations of antibiotics, a scenario which forces veterinarians to rely on research findings reported elsewhere (Morrow et al., 2020). Added to this, is the lack of official breakpoints for interpreting MIC values in avian mycoplasmas, which often complicates targeted treatment decisions (Hannan, 2000).

Additionally, culture and isolation still remain the gold standards for MG and MS detection, but this approach is time-consuming and always requires specialized knowledge and resources to implement. However, molecular detection methods such as polymerase chain reaction (PCR) are increasingly being used in diagnostic settings due to the fact that they offer faster and more sensitive alternatives (Khalifa et al., 2013; Amorim et al., 2024).

Despite these intensive control efforts, avian mycoplasmosis remains prevalent in Malaysian commercial poultry farms (Faisal et al., 2011). Additionally, molecular studies of pMGA and pvpA genes have revealed a significant genetic diversity among local MG isolates, with some exhibiting a high degree of similarity with vaccine strains like F and S6 (Yasmin et al., 2018; Taiyari et al., 2024). This genetic variation highlights the need for a targeted control efforts and continuous monitoring.

This study aims to determine the prevalence and patterns of antimicrobial susceptibility of isolates of MG and MS from commercial poultry farms in Central Peninsular Malaysia in light of these obvious concerns. The study intends to close important knowledge gaps, guide evidence-based treatment decisions, and aid in the development of focused, region-specific disease control strategies in order to protect poultry productivity and health by producing locally relevant data.

Materials and methods

Ethical approval

This study was approved by the Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia (UPM/IACUC/AUP-R031/2022)

Sample collection and study area

This study was conducted in the central region of Peninsular Malaysia comprising Selangor, Melaka, Negeri Sembilan, and Pahang states. A prevalence rate reported in a recent study in Malaysia (Taiyari et al., 2023) was used as the basis of sample size calculation and a minimum sample size of 359 was estimated according to (Thrusfield et al., 2018). However, a total of 407 choanal slit swab samples were collected from commercial poultry flocks during the study period, which spanned from March 2023 to December 2024. A stratified random sampling method was employed, ensuring inclusiveness across breeders ($n = 178$), layers ($n = 140$), broilers ($n = 70$), Deshi chicken ($n = 19$) regardless of the flock age or breed (Table 1). These samples were taken from different locations (Selangor, Melaka, Pahang and Negri Sembilan), from different farms and flocks within the farms to ensure variability and unbiased representation of the data. Moreover, samples were collected from flocks with or without vaccination histories, including birds with or without clinical signs. Also, birds selected for sampling had not been treated with antibiotics in the 21 days prior to sampling. Sterile plain swabs with plastic shafts, pre-moistened in Frey's mycoplasma medium, were used to aseptically collect samples from the choanal slit of each bird. After collection, the swabs were immediately placed on ice packs to maintain sample integrity and transported to the laboratory within 24 hours.

Table 1
Primers, primer sequences and size of the target genes used in the PCR assay.

Species	Target gene	Primer sequence (5′ to 3′)	Target size (bp)
M. gallisepticum	*mgc2*-F	TGAAGGTGAAACTAACTCCG	391
	mgc2-R	TCTTTGTTGGGGATTAGGAC	
M. synoviae	*vlhA*-F	TCAGCTACAGTTAGACTTGC	688
	vlhA-R	CAGGTCCAGTGTAGTTTAGG	

Isolation of Mycoplasma gallisepticum and Mycoplasma synoviae

Here, the standard protocol used for culture and isolation was (Dufour-Zavala, 2008).As soon as samples arrived at the laboratory, each swab sample was streaked on the agar surface and that same swab was dipped into a bijoux bottle containing 2 mL Frey mycoplasma broth. Broth cultures were then placed in the incubator with the temperature set at 37°C while the plates were placed in 5% humidified CO_2 incubator. Broth cultures were examined daily for color change from red to orange or yellow, while plates were first observed after 24 hrs of incubation for the presence of fast-growing mycoplasmas and then after every 2-3 days for the slow growing mycoplasmas which indicates the colonies are most likely MG or MS. Broth cultures turning yellow within the first 24 hours of incubation suggests an overwhelming presence of fast-growing mycoplasmas which will undoubtedly make the isolation of MG or MS difficult and challenging, and therefore, were discarded. Afterwards, any of the cultures that show color changes from red to orange or yellow were subcultured onto agar immediately, or after 1 week if there is no color change. Incubating agar plates were observed for up to 3 weeks after which they were discarded when no growth is observed. Single colonies of distinct mycoplasma colonies that were observed on agar plates after 3-5 days of incubation were picked and subcultured in broth. After incubation for color change, the broth culture was subjected to DNA extraction and PCR for confirmation. Positive MG or MS cultures were subjected to 3 rounds of subculturing on broth and agar to ensure purity of the isolates. Subsequently, PCR was performed on the presumed pure cultures and the positive PCR products were subjected to Sanger sequencing using the 16S universal primer to confirm their purity (Barghouthi, 2011).

DNA extraction

For molecular detection, twenty-four hours after incubation of the samples, 1mL of the initial broth cultures was pipetted into a 1.5 mL microfuge tube and subjected to the DNA extraction process using the boiling lysis method. Briefly, 1000 µl of the broth culture was centrifuged at 13,000 rpm for 10 min, the supernatant was then discarded. The pellet was resuspended with 100 µL of deionized distilled water. The mixture was then subjected to boiling lysis by incubating at 95°C for 10 min. Afterwards, the tubes were centrifuged at 13,000 rpm for 5 min and the supernatant which contains the released DNA was collected in new PCR tubes and stored at −20°C until needed for further analysis.

PCR amplification

A multiplex PCR was designed to detect MG and MS using the primer sequences of *mgc2* and *vlhA* genes respectively (Table 2). The multiplex PCR was optimized using REDiant II 2X mastermix (1st BASE,

Table 2
Distribution of choanal slit samples collected from commercial poultry farms in the central peninsular Malaysia.

LOCATION	BREED	NUMBER OF SAMPLES
Selangor	Deshi chicken	19
Selangor	Breeder	10
Selangor	Broiler	30
Pahang	Broiler	20
Melaka	Broiler	20
Selangor	Breeder	30
Negri Sembilan	Breeder	138
Selangor	Layer	32
Pahang	Layer	32
Melaka	Layer	35
Negri Sembilan	Layer	41
TOTAL		**407**

Singapore), 25 μL reaction mixture consisted of 12.5 μL mastermix, 1 μL *mgc2* forward primer (10 μM), 1 μL *mgc2 mgc2* reverse primer (10 μM),), 1 μL *vlhA* forward primer (10 μM), 1 μL *vlhA* reverse primer (10 μM), 5 μL template (DNA), and 3.5 μL sterile ddH_2O. Amplification was performed on a PCR instrument (Bio-Rad, Hercules, CA, USA) with the following thermal cycling conditions: an initial denaturation at 94 °C for 5 min followed by 30 cycles, each consisting of denaturation at 94 °C for 30 sec, annealing at 51°C for 30 sec, and extension at 72°C for 30 sec. The final stage of the reaction included an extension at 72 °C for 5 min in a thermal cycler. Subsequently, 5 uL DNA of each sample was then used to perform PCR amplification under the conditions stated above. Amplified DNA products were resolved on a 1.5% agarose gel in tris-acetic acid-ethylene diamine tetra acetic acid (EDTA-TAE) buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 8.3) for 40 minutes at a constant voltage of 100 V. Amplified products were detected with a gel imaging system (Bio-Rad). The amplified 16S DNA products of purified isolates were sequenced by Apical Scientific (Selangor, Malaysia). Sequencing results were compared using Blastn in the NCBI database.

Minimum inhibitory concentration (MIC) assay

Pure isolates of MG and MS were used to assess the MIC against antibiotics. Adopting the guidelines outlined by Hannan (2000), a microbroth dilution method was used to determine the MIC of antibiotics using a sensititre MIC plates precoated with nine (9) antibiotics used commonly in the treatment of avian mycoplasmosis. The pre-coated antibiotics used in this study were Tilmicosin (TIL), Tiamulin (TIA), Enrofloxacin (ENRO), Doxycycline (DOX), Erythromycin (ERY), Lincomycin (LIN), Chlortetracycline (CTET), Tylvalosin (TVN), Tylosin (TYLT). To assess the MIC of each strain, a stock culture was prepared by adding 1 mL of pure culture in 9 mL sterile broth and incubated at 37°C. Following incubation, cryoprotectant (glycerol) was added in a 5 % (V/V) dilution, aliquoted and then stored. Viability test was performed to achieve a standard inoculum size of 10^3 and 10^5 color-changing units per milliliter (ccu/mL) by pipetting 1 mL of the thawed stock culture and in 4 mL of sterile Frey's medium and the mixture was incubated at 37°C until color change was observed. Thereafter, 10-fold serial dilutions of the culture in smaller (1.5 mL) tubes was performed and the viable count followed by the addition of 100 μL of sterile Frey's medium to 8 consecutive wells in a single row of a 96-well plate. Subsequently, 100 μL of each corresponding serial dilution of the culture was added to each well, with the highest dilution placed in well 1 and the lowest dilution placed in well 8. In well number 11 (positive control), 100 μL of sterile medium and 100 μL of stock culture were added, while in well number 12 (negative control), 200 μL of sterile medium was added and the viable count plate was then incubated at 37°C. The plates were incubated for 1-2 weeks until a complete color change was observed. Results of the viable plate count were recorded as the lowest dilution with no further colour change following incubation, denoting the reciprocal of the number of colour changing units (ccu) per mL. Once the inoculum size has been standardized, the determination of the MIC followed immediately by dispensing 50uL of each isolate on all the wells of the Sensititre MIC microtiter plate which was later sealed and incubated at 37°C. The plates were then monitored until a complete color change in the positive control well of the MIC plate was observed. At that point, the MIC plate was placed on a white background and the MIC for each antibiotic was determined as the well with the lowest concentration of antibiotic that produces no color change. Note that, in each of the tests carried out to assess the MIC for either MG or MS strain, positive control was included thus, MG (MG ATCC 19610) and MS (MS ATCC 25204).

Results

Isolation of MG and MS

In the present study, multiplex PCR and subsequently culture and isolation were used to detect and determine the prevalence of MG and MS. A total of 407 birds (178 breeders, 140 layers, 70 broilers, 19 Deshi chicken) were sampled and subjected to PCR. An overall total of 103 (25.3 %) MG, 173 (42.5 %) MS and 36 (8.8 %) MG-MS co-infection were obtained by PCR. Out of 70 broilers, 29 (41.4 %) were found to be positive for MG and 7 (10 %) positive for MS; out of 140 layers, 29 (20.7 %) were positive for MG and 67 (47.9 %) were positive for MS; Out of 178 breeders, 43(24.3 %) were found to be positive for MG and 81 (45.5 %) were positive for MS; out of 19 Deshi chicken sampled, 2 (10.5 %) were positive for MG and 18 (94.7 %) were positive for MS. Meanwhile, 62(15.2 %), 14(3.4 %) and 1 (0.2 %) MG-MS successful isolation of MG, MS and MG-MS co-infection were made respectively. Results of isolation and PCR detection are shown in (Table 3). DNA bands of expected sizes were observed both in the case of MG (391 bp) and MS (688 bp) (Fig. 1). Pure cultures of MG and MS were subjected to sequencing. The DNA sequencing results of the PCR products were subjected to BLAST analysis for confirmation.

Minimum inhibitory concentration (MIC) assay

MIC was performed on purified MG and MS isolates obtained from field samples. (Table 4) shows MG strains were found to show low MIC values (≤0.12 μg/mL) against Tilmicosin (TIL), Tiamulin, Enrofloxacin (ENRO), Doxycycline (DOX), Erythromycin (ERY), and Tylosin (TYLT) which therefore suggest high susceptibility of the strains to the tested antibiotics. MIC values for Lincomycin (LIN) were found to be in the range of 4 to 8 μg/mL, thus suggesting a reduced susceptibility. Incidentally, the MIC values for Chlortetracycline (CTET) were seen to have shown some variation from 1 to 8 μg/mL, which could suggest having variable susceptibility among the tested strains. For all the MG isolates, the MIC values for Tylvalosin (TVN) were found to be low (≤0.06 μg/mL), which might a be a good indication of its remarkable efficacy against MG strains. On the other hand, the strains of MS strains tested (Table 5) showed remarkable high MIC values for tilmicosin (TIL) and erythromycin (ERY) especially when compared with the reference strain which may indicate reduced susceptibility or potential resistance. Meanwhile, the MIC values for tiamulin (TIA) were observed to be relatively low (0.12 - 0.5 μg/mL) which is an indication of a maintained efficacy. Enrofloxacin (ENRO) and doxycycline (DOX) showed moderate MIC values, with some variability among strains. Meanwhile, high MIC values for lincomycin (LIN) and chlortetracycline (CTET) in all the isolates were observed which may as well suggest some potential resistance.

Discussion

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are important pathogens of poultry, capable of causing significant economic losses worldwide (Kursa et al., 2024). MG and MS infections have a negative impact on poultry health and productivity. This results in

Table 3
Prevalence of Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS) from samples obtained from the central peninsular Malaysia.

LOCATION	BREED	SAMPLED	PCR			CULTURE		
			MG	MS	MG MS	MG	MS	MG MS
Selangor	Deshi chicken	19	2	18	ND	NR	NR	NR
Selangor	Breeder	10	5	ND	ND	4	NR	NR
Selangor	Broiler	30	22	1	ND	NR	NR	NR
Pahang	Broiler	20	7	ND	ND	1	NR	NR
Melaka	Broiler	20	ND	6	ND	NR	10	NR
Selangor	Breeder	30	7	ND	ND	NR	NR	NR
Negri Sembilan	Breeder	138	31	81	15	38	3	NR
Selangor	Layer	32	ND	ND	ND	NR	NR	NR
Pahang	Layer	32	2	11	4	5	NR	NR
Melaka	Layer	35	22	24	15	8	NR	NR
Negri Sembilan	Layer	41	5	32	2	5	1	1
		407	**103(25.3 %)**	**173 (42.5 %)**	**36(8.8 %)**	**61(15 %)**	**14(3.4 %)**	1(0.2 %)

*MGMS = Co-infection of MG and MS, ND = No detection, NR = No recovery.

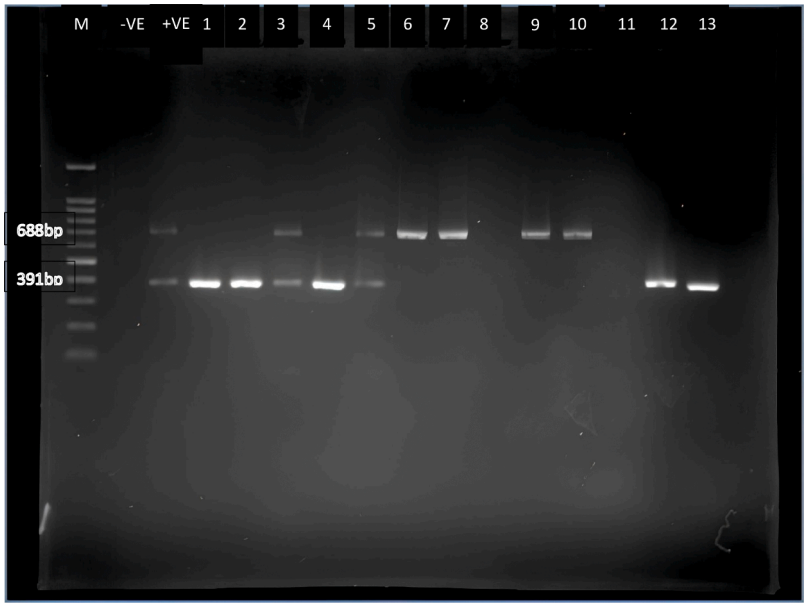

Fig. 1. Agarose gel image showing results of the multiplex PCR used to detect *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) from field samples.
Note: *M* = 100 bp ladder; -VE = Negative control; +VE = Positive control; Lane 1, 2, 4, 12 & 13 = MG positive; Lane 6, 7, 9 & 10 = MS positive; Lane 3 & 5 = MG & MS positive (Mixed infection); Lane 8 & 11 = negative samples.

reduced feed conversion efficiency, poor weight gain and increased mortality. In addition, these pathogens can result in trade restrictions arising from condemnation of carcasses of infected flocks, leading to even greater economic losses. Proper implementation of biosecurity measures, judicious use of antibiotics and routine vaccination have been repeatedly used to limit the impact of these infections (Kleven, 2008; El-Ashram et al., 2021; Mugunthan et al., 2023; Kursa et al., 2024). Determination of antibiotic susceptibility of mycoplasmas is laborious, time-consuming and often requires special techniques to perform, thus, it is not performed routinely (Hannan, 2000). This factor has led poultry farmers to misuse antibiotics without recourse to concerns for the development of antibiotic-resistant strains (Gautier-Bouchardon, 2018); therefore, regular monitoring of the MIC of antibiotics is essential to know the susceptibility of these pathogens to different antimicrobial agents as it will assist in choosing effective treatment options and adjusting therapeutic strategies to address antibiotic resistance concerns.

In the present study, an overall total of 103 (25.3 %) MG, 173 (42.5 %) MS and 36 (8.8 %) MG-MS were obtained by PCR, while 61 (15.%) 14 (3.4 %) and 1 (0.2 %) successful isolation of MG, MS, and MG-MS were made respectively. These findings agree with the findings earlier reported (Muhammad et al., 2018; Chaidez-Ibarra et al., 2022). It has long been established that PCR is a much faster and sensitive method of diagnosing avian mycoplasmosis. However, the role of culture as the gold standard for diagnosis remains relevant. This fact is reinforced by the indispensable role of isolation in determining the MIC of antibiotics, which is critical in monitoring the potential development of antibiotic resistance (García et al., 2005; Muhammad et al., 2018).

Incidentally, the higher prevalence of MS observed in the present study in comparison with MG, agrees with a recent study conducted in Malaysia (Taiyari et al., 2024), which differs with earlier findings reported in the country (Yamamoto et al., 1992) and South Asia (Chaidez-Ibarra et al., 2022). This changing trend in the prevalence of these organisms as observed by our findings is key, as researchers seek to understand and implement strategies aimed at combating the impact of the disease. Although both pathogens (MG and MS) remain relevant to the poultry industry (Feberwee et al., 2022), researchers in the field have long held the view that the overall impact of MG is greater than MS (Landman, 2014); this assessment is based on several factors, such as the disease severity, economic impact, and pathogenicity (Mohammed et al., 1987; Bergeron et al., 2021; WOAH, 2021). The reason proposed here is that MS was associated mainly with subclinical respiratory

Table 4
Antimicrobial minimum inhibitory concentration (MIC) values of *Mycoplasma gallisepticum* (MG) field isolates and reference strain.

Mycoplasma gallisepticum										
MIC (µg/mL)										
Strain	**Location**	**TIL**	**TIA**	**ENRO**	**DOX**	**ERY**	**LIN**	**CTET**	**TVN**	**TYLT**
MG ATCC 19610*	NA	≤0.06	≤0.015	≤0.03	≤0.06	≤0.12	4	2	≤0.03	≤0.06
UPMMG3	Melaka	≤0.06	≤0.015	0.06	≤0.06	≤0.12	8	2	0.06	≤0.06
UPMMG4	Melaka	≤0.06	≤0.015	≤0.03	≤0.06	≤0.12	8	1	0.06	≤0.06
UPMMG5	Melaka	≤0.06	≤0.015	≤0.03	≤0.06	≤0.12	8	1	0.06	≤0.06
UPMMG8	Melaka	≤0.06	≤0.015	≤0.03	0.12	≤0.12	8	2	0.06	≤0.06
UPMMG9	Melaka	0.12	0.06	0.12	≤0.06	≤0.12	8	2	≤0.015	0.12
UPMMG6	Melaka	≤0.06	≤0.015	0.12	0.12	≤0.12	8	4	≤0.015	≤0.06
UPMMG7	Melaka	≤0.06	≤0.015	0.12	0.12	≤0.12	8	8	0.06	≤0.06
UPMMG10	Melaka	≤0.06	≤0.015	0.06	≤0.06	≤0.12	4	2	0.03	≤0.06
UPMMG11	Melaka	≤0.06	≤0.015	0.12	0.12	≤0.12	4	2	0.03	≤0.06
UPMMG12	Negeri Sembilan	≤0.06	≤0.015	0.12	0.12	≤0.12	4	4	0.06	0.12
UPMMG13	Negeri Sembilan	≤0.06	≤0.015	0.12	0.12	≤0.12	8	1	0.06	≤0.06
UPMMG15	Negeri Sembilan	≤0.06	≤0.015	0.12	0.12	≤0.12	8	1	0.03	≤0.06
UPMMG16	Negeri Sembilan	≤0.06	≤0.015	0.12	0.12	≤0.12	8	1	0.03	≤0.06
UPMMG14	Negeri Sembilan	≤0.06	≤0.015	0.12	0.12	≤0.12	4	2	0.06	≤0.06
UPMMG2	Pahang	≤0.06	≤0.015	0.12	0.12	≤0.12	8	1	0.06	≤0.06
UPMMG1	Pahang	≤0.06	≤0.015	0.06	0.12	≤0.12	8	1	0.06	0.12

Reference strain (*), NA (Not available), Tilmicosin (TIL), Tiamulin (TIA), Enrofloxacin (ENRO), Doxycycline (DOX), Erythromycin (ERY), Lincomycin (LIN), Chlortetracycline (CTET), Tylvalosin (TVN), Tylosin (TYLT).

Table 5
Antimicrobial minimum inhibitory concentration (MIC) values of *Mycoplasma synoviae* (MS) field isolates and reference strain.

Mycoplasma synoviae **MIC (µl/mL)**										
Strain	Location	TIL	TIA	ENRO	DOX	ERY	LIN	CTET	TVN	TYLT
MS ATCC 25204*	NA	0.12	0.06	0.05	0.25	32	1	2	0.03	0.5
UPMMS5	Melaka	64	0.25	1	0.12	≥64	≥32	1	0.25	2
UPMMS8	Melaka	64	0.25	4	0.25	≥64	≥32	4	0.5	4
UPMMS2	Melaka	32	0.12	2	0.12	≥64	≥32	1	0.5	2
UPMMS4	Melaka	32	0.25	2	0.25	≥64	≥32	2	0.5	4
UPMMS10	Melaka	32	0.25	2	0.25	≥64	≥32	8	0.5	4
UPMMS6	Melaka	64	0.5	2	0.5	≥64	≥32	8	0.5	8
UPMMS59	Melaka	32	0.12	0.5	0.25	32	≥32	2	0.5	2
UPMMS7	Melaka	64	0.5	2	0.5	≥64	≥32	8	0.5	8
UPMMS3	Melaka	64	0.5	4	0.25	≥64	≥32	4	0.25	2
UPMMS1	Negeri Sembilan	32	0.25	4	0.25	≥64	≥32	4	0.5	4
UPMMS11	Negeri Sembilan	32	0.25	4	0.25	≥64	≥32	4	0.5	8
UPMMS12	Negeri Sembilan	32	0.25	4	0.25	≥64	≥32	4	0.5	4

Reference strain (*), NA (Not available), Tilmicosin (TIL), Tiamulin (TIA), Enrofloxacin (ENRO), Doxycycline (DOX), Erythromycin (ERY), Lincomycin (LIN), Chlortetracycline (CTET), Tylvalosin (TVN), Tylosin (TYLT).

infections and was considered to have a lower clinical and economic impact on broilers ((Feberwee et al., 2009). However, recent indicators, both from clinical and economic standpoints, along with the emergence of strains affecting eggshell quality and egg production (Feberwee et al., 2009), coupled with an increasing prevalence of the organism as reported in recent studies, all seem to suggest the growing relevance of MS in the poultry industry (Landman, 2014). Additionally, the high sero-prevalence of MS over MG as observed in countries with well-established poultry industry, and its ability to interact with other pathogens, further adds to the significance of MS in the poultry industry (Rehman, 2018).

Determination of MIC in the present study involved the inclusion of reference strains MG ATCC 19610 and MS ATCC 25204 as controls. The values of MIC obtained from the reference strains in the present study aligns with values reported by (Hannan, 2000), thus implying that our MIC test was valid. In the present study, Tylvalosin was found to be the most effective antibiotic to treat avian mycoplasmosis especially in instances where co-infection of MG and MS exist as highlighted in the present study. Interestingly, Tylvalosin is a recent introduction in the poultry industry in Southeast Asia and this may explain why significantly low MIC values of the drug were observed (Morrow et al., 2020). Although several factors could account for the low MIC values observed in Tylvalosin, one of such factors is that newer antibiotcs are designed to target specific pathogens or mechanisms of action which ensures their efficacy at low concentrations and also less likely to develop resistance (Miethke et al., 2021). Findings from this study agree with similar observations in Thailand, Egypt, Iran, and Europe (Pakpinyo and Sasipreeyajan, 2007; Grózner et al., 2016; Kreizinger et al., 2017; Abd El-Hamid et al., 2019; Zhang et al., 2022; Limpavithayakul et al., 2023; Taiyari et al., 2024). Consistently low MIC values observed for Tiamulin suggests that it remain effective against MG and MS, invariably corroborating the findings from a recent study (Wang et al., 2022). This finding is also supported by earlier studies conducted (Garmyn et al., 2017; Abonashey et al., 2024; Taiyari et al., 2024). It has also been found that MG and MS do not develop resistance after 10 passages in a sub-inhibitory concentration of Tiamulin (Nhung et al., 2016). Lincomycin was observed to have high MIC values for both MG and MS. Similar observation was made in Italy (Bottinelli et al., 2022). Among the lincosamides, Lincomycin is the only drug approved for use in poultry and has been proven to have an enhanced activity against avian mycoplasmosis in combination with spectinomycin (Hamdy et al., 1982; Baggot and Giguère, 2013). Usually, resistance to lincosamides manifest as cross-resistance to macrolides, lincosamides and streptogramin B antibiotics, termed MLSB resistance. This phenomenon occurs due to the use of these antibiotics at the same time since they have similar binding sites in the bacterial 50S ribosomal subunit. In particular, the

macrolide binding site overlaps that of lincosamides, resulting in cross-resistance (Leclercq, 2002) Tilmicosin, a semisynthetic macrolide antibiotic, has shown excellent antibacterial effect against Mycoplasmas (Kempf, Gesbert, et al., 1997). However, due to indiscriminate use of antibiotics, reduced susceptibility of this class of antibiotic against MS has been observed (Kempf et al., 1997; Gautier-Bouchardon, 2018; Yan et al., 2023), this observation could possibly explain the reason why reduced susceptibility of MS against. Tilmicosin was observed in the present study, although a remarkable activity against MG was observed. Also in the present study, high MIC values for Enroflaxacin against MS were observed, which agrees with findings from a previous study earlier conducted in Italy, Thailand and Malaysia (Catania et al., 2019; Limpavithayakul et al., 2023; Taiyari et al., 2024). Similar studies conducted in Israel and Europe reportedly described Enrofloxacin resistance in MS, where 59 % of field MS strains tested out of 179 were found to have a reduced susceptibility to enroflaxacin MIC >1 to > 16 μg/mL) (Lysnyansky et al., 2013). Similarly, a study of 32 MS isolates recently conducted in China from 2016 to 2019 also found high values for the MIC for Enrofloxacin, with a range of 4–32 μg/mL, impliedly suggesting that enrofloxacin resistance is prevalent in Chinese MS strains (Zhang et al., 2022). The emergence of resistance to enrofloxacin in MS has been associated mainly by mutations in the quinolone resistance-determining regions (QRDRs) of genes coding for DNA gyrase and topoisomerase IV. Specific mutations within the parC gene, such as C254T, have also been incriminated with reduced susceptibility. Moreover, amino acid alterations such as Glu804Gly and Thr686Ala through mutations in the gyrA gene have previously been detected in resistant isolates. In a similar study, a correlation between amino acid substitutions in the parC QRDR such as Asp79Asn, Thr80Ala/Ile, Ser81Pro, and Asp84Asn/Tyr/His, and decreased susceptibility to enrofloxacin was highlighted. Mutations were also detected at positions GyrA 87, GyrB 401/402, and ParE 420/454, but it was uncertain whether these observations directly contribute to resistance (Le Carrou et al., 2006; Lysnyansky et al., 2013; Gautier-Bouchardon, 2018; Bekő et al., 2020)

Erythromycin, a macrolide antibiotic was observed to have high MIC values in the present study. Our observation seems to be in agreement with the findings earlier reported in Egypt, Italy, Iran and Malaysia (Ghaleh Gol et al., 2008; Abd El-Hamid et al., 2019; Catania et al., 2019; Taiyari et al., 2024). MS resistance against macrolides has been reported in previous studies (Kleven and Anderson, 1971; Whithear et al., 1983; Bradbury et al., 1994; Gautier-Bouchardon et al., 2002; Kreizinger et al., 2017) suggesting a mechanism of natural resistance, as already observed in *Mycoplasma hominis* (Furneri et al., 2000). Reduced susceptibility characterized by high MIC value for chlortetracycline was equally observed in this study, which is consistent with findings previously reported in Thailand, Egypt and recently in Malaysia (Pakpinyo and Sasipreeyajan, 2007; Abd El-Hamid et al., 2019; Taiyari et al., 2023, 2024). Chlortetracycline was introduced in the poultry industry a longtime ago as a drug of choice in the management of avian mycoplasmosis, this, along with its widespread use, is believed to be the reason for these observations (Pakpinyo and Sasipreeyajan, 2007)

Conclusions

In conclusion, the prevalence of MS seems to be higher than MG, this finding is in agreement with recent observation of higher prevalence of MS in Southeast Asia although earlier studies reported otherwise. It reflects an emerging epidemiological trend of avian mycoplasmosis within Malaysia. Tylvalosin seems to be an excellent choice for both MG and MS, which, despite MG still responding to several antibiotics, a markedly reduced susceptibility to several of the antibiotics was noted for MS suggesting an emerging antibiotic resistance. Thus, we argue for routine monitoring of these organisms along with antimicrobial susceptibility testing to guide treatment options and also minimize potential economic losses and as well address potential concerns regarding antimicrobial resistance.

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