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Genetic Variability and Antimicrobial Susceptibility Profile of *Mycoplasma gallisepticum* and Antimicrobial Susceptibility Profile of *Mycoplasma synoviae* Isolated from Various Bird Species in Peninsular Malaysia

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ABSTRACT

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) can infect many bird species. Susceptible species of birds are responsible for spillover infections from poultry to wild birds. In Malaysia, previous studies reported the detection of MG in free-flying birds, but there is a lack of information on the characterization and antimicrobial susceptibility profile of these isolates. Therefore, this study aims to molecular characterize and assess the minimum inhibitory concentration (MIC) of MG and MS isolated from different bird species, including aviary, free-flying, and pet birds. Altogether 54 choanal slit swab samples were collected. All the swab samples were subjected to isolation and polymerase chain reaction (PCR) detection. Positive samples were subjected to molecular characterization and MIC assay. Molecular characterization was done using targeted gene sequencing, and microdilution MIC assay was employed to determine the antimicrobial susceptibility profile of the isolates. Using both culture and PCR techniques, 5.6% (3/54) of the samples were positive for MG, while MS was detected in 29.6% (16/54) and 40.7% (22/54) of samples using culture and PCR, respectively. The partial sequence of the *mgc2* gene showed a distinct pattern of this gene in one of the MG isolates. Phylogenetic analysis of this isolate indicated that this isolate is a progeny of a Thailand poultry strain. Microdilution MIC assay indicated

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100% resistance to erythromycin and chlortetracycline. The findings of this study indicate the possibility of avian interspecies transmission of MG and MS in Malaysia and the need for antimicrobial treatment dosage optimization.

Keywords: Bird species, microdilution, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, targeted gene sequencing

INTRODUCTION

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) are among the most significant mycoplasmal infections of birds (Friend, 1999; Luttrell & Fischer, 2007). Historically, MG and MS were thought to be host-specific and pathogenic bacteria primarily for poultry species (Raviv & Ley, 2013). However, the identification of MG as the causative agent of conjunctivitis outbreak in house finch (*Haemorhous mexicanus*) gave a new perspective on the pathogenicity of MG (Taiyari & Abu, 2020). The MG infection in house finch spread rapidly, and throughout its expansion, host jump events were reported among other passerine species (Dhondt et al., 2014). *Mycoplasma gallisepticum* was also reported to cause an outbreak in the wild turkey population (Davidson et al., 1982). Apart from wild turkey and house finch, MG and MS have been isolated from several species of free-ranging birds including pheasants, chukar partridge, grey partridge, peafowl, Bobwhite quail, Japanese quail, ducks, geese, yellow-naped Amazon parrot, greater flamingos, American goldfinches, pine grosbeaks, evening grosbeaks, purple finch, blue jay, pigeon, and red-legged partridge (Ferguson-Noel & Noormohammadi, 2013; Raviv & Ley, 2013). Identifying new natural hosts plays a crucial role in comprehending transmission routes and carriers of these important pathogens. From the past to the present, MG and MS infections have occurred in high numbers among poultry farms in Malaysia (Yasmin et al., 2014). A previous study in Malaysia detected MG in crows by polymerase chain reaction (PCR) (Ganapathy et al., 2007). It indicates the possible role of free-flying birds in the circulation and transmission of MG in Malaysia.

MG and MS infections are detected via culture, serological, and molecular techniques. The culture technique includes the isolation and subsequent identification step using immunofluorescence staining. Although the culture technique is laborious and time-consuming, its capability to identify all mycoplasma species with high specificity is still paramount (Levisohn & Kleven, 2000). PCR offers a rapid and convenient diagnosis of pathogenic mycoplasmas. However, few studies reported the false diagnosis of MG by PCR (Ganapathy & Bradbury, 1999; Kempf et al., 1997). The products of PCR can be used for genotyping and phylogenetic analysis of the isolates. Gene-targeted sequencing (GTS) is a reproducible typing method with satisfactory discriminatory power to separate the isolates (Ferguson et al., 2005). The *mgc2* gene of MG is responsible for encoding major surface proteins with pathogenic, antigenic, and immune evasion properties (Markham et al., 1993). Since different strains of MG have varied sizes of

the *mgc2* gene and only a single copy of this gene can be found in the MG genome, this gene was selected for genotyping of isolates in this study (Bencina, 2002).

Good biosecurity and consistent monitoring programs allow the poultry industry to control and prevent mycoplasmosis. Although these preventive programs have been successful in some aspects, the complete control of avian mycoplasmosis seems difficult due to large poultry populations, multi-age flocks, and unidentified reservoirs (Kleven, 2008). In addition, studies have shown that MG can survive in the environment longer than was previously assumed (Shah-Majid, 1988). This finding adds to the difficulties of controlling mycoplasmosis. Antibiotic treatment may be very beneficial and effective in reducing clinical signs and disease transmission, but the long-term use of antimicrobial agents may develop resistance (Reinhardt et al., 2005). Therefore, although antibiotic medications could prevent economic losses related to avian mycoplasmosis, these prevention programs should be considered for short periods (Kleven, 2008). Macrolides tend to be effective against avian mycoplasmosis (Kleven, 2008). Macrolides such as tylosin and erythromycin are Malaysia's first antibiotic therapy choice against mycoplasmosis. However, there is a lack of information on the antimicrobial minimum inhibitory concentration (MIC) of Malaysian MG and MS isolates. The guideline to conduct MIC tests for pathogenic avian mycoplasmas was described by Hannan (2000).

Therefore, this study aims to investigate the sustainability of MG and MS infections in non-poultry birds by characterizing the field isolates using molecular techniques and determining antimicrobial susceptibility profiles.

METHODS

Samples Descriptives

A total of 54 choanal slit swab samples were collected from various species of birds without clinical signs of avian mycoplasmosis. Some of these birds were kept as aviary birds on an ornamental farm. Aviary birds were inclusive of jungle fowl, guinea fowl, and peacock. The other group of birds was free-flying, including raptors and non-raptor species. Non-raptor species were caught using a mist net at an ornamental bird farm in Selangor, Malaysia. Pigeons and spotted doves were the non-raptor species. The public donated raptors to the University Veterinary Hospital (UVH) for rehabilitation. Barn owls, Asian brown wood owls, crested serpent eagles, and buffy fish owls were the raptor species. The rest of the birds were pet birds that were admitted to UVH. Budgerigar and mynah were the pet species. Descriptive of different bird categories are presented in Table 1. This study was approved by the Institutional Animal Care and Use Committee (IACUC) (UPM/IACUC/AUP-R069/2019).

Capture of Free-flying Birds

Free-flying birds were captured at the ornamental farm's site. Briefly, a mist net was set up and placed between the trees for six hours, which was done in the morning. Every twenty minutes, the trap was checked

Table 1
Descriptive of different bird categories

Category	Species of bird	No.
Aviary birds	Jungle fowl (*Gallus gallus*)	30
	Peafowl (*Pavo cristatus*)	3
	Guineafowl (*Numida meleagris*)	6
Free flying birds	Pigeon (*Columba livia domestica*)	5
	Spotted dove (*Spilopelia chinensis*)	2
	Barn owl (*Tyto alba*)	1
	Asian brown wood owl (*Strix leptogrammica*)	1
	Crested serpent eagle (*Spilornis cheela*)	1
	Buffy fish owl (*Ketupa ketupu*)	1
Pet birds	Budgerigar (*Melopsittacus undulatus*)	2
	Common myna (*Acridotheres tristis*)	2

for the presence of any bird. The trapped birds were restrained properly (Bailey, 2016) and subjected to sample collection.

Isolation Technique

Frey medium with 15% swine serum (FMS) (Merck, Germany) was prepared according to the literature (Ferguson-Noel & Kleven, 2016). A cotton swab with a plastic shaft was pre-wet with FMS prior to sample collection. Once the sample was collected for each bird, the swabs were kept inside individual FMS bottles and transported to the laboratory inside a cool box within 24 hr. Upon arrival at the laboratory, the bottles were incubated at 36°C. After 24 hr of incubation, broth cultures were subjected to FMS agar inoculation and PCR. Agar plates were incubated at 36°C under carbon dioxide (CO_2) conditions by putting them inside candle jars. Frey medium with 15% FMS agar inoculation was repeated for each sample when the broth cultures showed a color change from red to orange/yellow. Plates were monitored every three days under a stereo microscope for any sign of mycoplasma colonies.

According to the literature, the mycoplasma colonies were identified using immunofluorescence assay (IFA) (Ferguson-Noel & Kleven, 2016). A block of agar (0.5 cm × 0.5 cm) containing mycoplasma colonies was cut from each sample and subjected to IFA. For identification of MG colonies, a high-tittered MG-specific rabbit polyclonal antibody (IgG) conjugated with Alexa fluor 488 (Bioss Antibodies, USA [diluted with phosphate-buffered saline (PBS) 1:200]) was used in direct IFA. An indirect immunofluorescence technique was used to identify MS colonies (Ferguson-Noel & Kleven, 2016). This technique requires a specific primary antibody with the ability to detect MS. In addition, there should be a secondary antibody conjugated with fluorescein and capable of attaching to the primary antibody. This study used a diluted MS-specific chicken

polyclonal antibody (IgY) as the primary antibody (Abcam, United Kingdom). For the secondary antibody, goat anti-chicken IgY conjugated with phycoerythrin (PE) (Abcam, United Kingdom) was used. Stained agar blocks were observed under a fluorescent microscope.

DNA Extraction

For molecular detection, twenty-four hours after incubation of the samples, 1 ml of the initial broth cultures was pipetted into a 1.5 ml tube and subjected to the DNA extraction process using QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer's recommendations. Briefly, 20 µl of proteinase K and 200 µl of AL buffer were added to the plaque, and then the mixture was vortexed for about 20 s to detach the plaque from the bottom of the tube. After that, the tube was incubated at 56°C for 1 hr. The tube was vortexed several times during the incubation period. After the incubation, 200 µl of 99% alcohol (Labchem, Malaysia) was added to the tube and vortexed for about 20 s. The entire tube contents were transferred to a spin column and then centrifuged at 6,000 × *g* for 1 min. The collection tube was discarded, and the spin column was placed on a new collection tube. About 500 µl of buffer AW1 was added to the spin column and centrifuged at 6,000 × *g* for 1 min. The collection tube was discarded, and the spin column was placed on a new collection tube. Approximately 500 µl of buffer AW2 was added to the spin column and centrifuged at 20,000 × *g* for 5 min. After discarding the collection tube, the spin column was placed on a 1.5 ml tube. Finally, 100 µl of buffer AE was added to the spin column, incubated at room temperature for 3 min, and centrifuged at 6,000 × *g* for 1 min to elute the DNA.

Polymerase Chain Reaction (PCR)

Universal 16S rRNA primers were used to detect MG and MS in multiplex PCR assay (Ferguson-Noel & Kleven, 2016; Moscoso et al., 2004; Nicholas & Baker, 1998). Two sets of primers targeting 16S rRNA gene of MG (MG-16S rRNA F:5′-GAC CTA ATC TGT AAA GTT GGT C-3′; MG-16S rRNA R:5′-GCT TCC TTG CGG TTA GCA AC-3′) and MS (MS-16S rRNA F:5' -GAG AAG CAA AAT AGT GAT ATC A-3'; MS-16S rRNA R:5' -CAG TCG TCT CCG AAG TTA ACA A- 3') were used. The reaction solution was prepared at 25 µl. Initial denaturation was at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A final extension was performed at 72°C for 5 min. Notably, ATCC 19610 and ATCC 25204 were positive controls in gel electrophoresis to detect MG and MS isolates, respectively.

Phylogenetic Analysis

Molecular characterization was done by targeting the partial sequence of the MG *mgc2* gene (Moscoso et al., 2004). Positive MG colonies were subjected to DNA extraction using QIAamp DNA Mini Kit (Qiagen, USA). The DNA extracts were then subjected to PCR assay for the MG *mgc2* gene. Initial denaturation was at 94°C for 5 min, followed by 35 cycles of

94°C for 30 s, 58°C for 30 s, and 72°C for 60 s, and final extension was performed at 72°C for 5 min. The PCR products were observed by gel electrophoresis. Samples that showed distinguished *mgc2* band size compared to the MG positive control were selected for targeted sequencing. Using CLUSTALW, the sequences of the isolates and positive controls were aligned. Percentage differences among sequences were calculated, and then a phylogenetic tree was constructed using MEGA7.

Antimicrobial Susceptibility Profile

The antimicrobial susceptibility profile of the isolates was determined according to Hannan (2000) as well as Tanner and Wu (1992). The antibiotics used in this study were inclusive of tilmicosin, tiamulin, enrofloxacin, doxycycline, erythromycin, lincomycin, chlortetracycline, tylvalosin, and tylosin. After preparing pure cultures, viable counting was performed to achieve the 10^4 color-changing units inoculum size of MG and MS isolates. Briefly, 10-fold serial dilutions of the stock cultures were made. From each serial dilution, 100 µl was pipetted in 100 µl of FMS in a 96-well plate (viable counting plate). The viable counting plate was incubated at 37ºC with the positive control tube containing one ml of stock culture and 9 ml of sterile FMS. The viable counting plate was monitored for color change three times per day. Once the 10^{-3} well of viable counting plate changed color from red to orange, the positive control tube of the same stock culture was subjected to MIC plate inoculation. After inoculating 100 µl of positive control into each MIC plate well, the MIC plate was incubated at 37ºC. A schematic view of the MIC plate is shown in Figure 1. The MIC plates were monitored

SENSITITRE
CMP1VEAH
Veterinary Reference Card

	1	2	3	4	5	6	7	8	9	10	11	12
A	TIL 0.06	TIL 0.12	TIL 0.25	TIL 0.5	TIL 1	TIL 2	TIL 4	TIL 8	TIL 16	TIL 32	TIL 64	TIL 128
B	TIA 0.015	TIA 0.03	TIA 0.06	TIA 0.12	TIA 0.25	TIA 0.5	TIA 1	TIA 2	TIA 4	TIA 8	TIA 16	TIA 32
C	ENRO 0.03	ENRO 0.06	ENRO 0.12	ENRO 0.25	ENRO 0.5	ENRO 1	ENRO 2	ENRO 4	ENRO 8	ENRO 16	ENRO 32	DOX 0.06
D	DOX 0.12	DOX 0.25	DOX 0.5	DOX 1	DOX 2	DOX 4	DOX 8	DOX 16	ERY 0.12	ERY 0.25	ERY 0.5	ERY 1
E	ERY 2	ERY 4	ERY 8	ERY 16	ERY 32	ERY 64	LIN 0.12	LIN 0.25	LIN 0.5	LIN 1	LIN 2	LIN 4
F	LIN 8	LIN 16	LIN 32	CTET 0.25	CTET 0.5	CTET 1	CTET 2	CTET 4	CTET 8	CTET 16	TVN 0.015	TVN 0.03
G	TVN 0.06	TVN 0.12	TVN 0.25	TVN 0.5	TVN 1	TVN 2	TVN 4	TVN 8	TVN 16	TVN 32	TVN 64	TVN 128
H	TYLT 0.06	TYLT 0.12	TYLT 0.25	TYLT 0.5	TYLT 1	TYLT 2	TYLT 4	TYLT 8	TYLT 16	TYLT 32	TYLT 64	POS

Figure 1. Schematic view of the customized sensititre plate used in this study (numbers inside each well indicate the antibiotic concentration in µg per ml)
Note. TIL = Tilmicosin; TIA = Tiamulin; ENRO = Enrofloxacin; DOX = Doxycycline; ERY = Erythromycin; LIN = Lincomycin; CTET = Chlortetracycline; TVN = Tylvalosin; TYLT = Tylosin; POS = Positive control

three times per day. Once the positive well of the MIC plate changed color from red/orange to yellow, the MIC plates were taken out from the incubator, and the initial MIC was recorded.

RESULTS

Detection of *M. gallisepticum* and *M. synoviae*

In the samples collected from aviary birds, MG was detected in 7% (3/41) of samples using both culture and PCR techniques. In aviary birds, 44% (18/41) and 32% (13/41) of samples had MS using PCR and culture, respectively.

In free-flying birds, 27% (3/11) and 36% (4/11) of the samples were positive for MS by culture and PCR, respectively. No MG was detected in the samples of free-flying birds. No MG and MS were detected in pet bird samples. Figure 2 demonstrates the multiplex PCR agarose gel result indicating the detection of MG and MS in some PCR products.

Phylogenetic Analysis of Isolates

After amplifying the *mgc2* gene using the strain-specific primers, the PCR product was sent for purification and gene-targeted sequencing. Figure 3 shows the gel electrophoresis result of the MG isolate PCR product using *mgc2* strain-specific primers. A total of 3 MG isolates were made in this study, but only one of them, which was isolated from a jungle fowl (accession number: ON645899), showed different *mgc2* band sizes in comparison to the positive control. The sequencing coverage of this gene was 269 bp. This sequencing coverage was used to construct a phylogenetic tree.

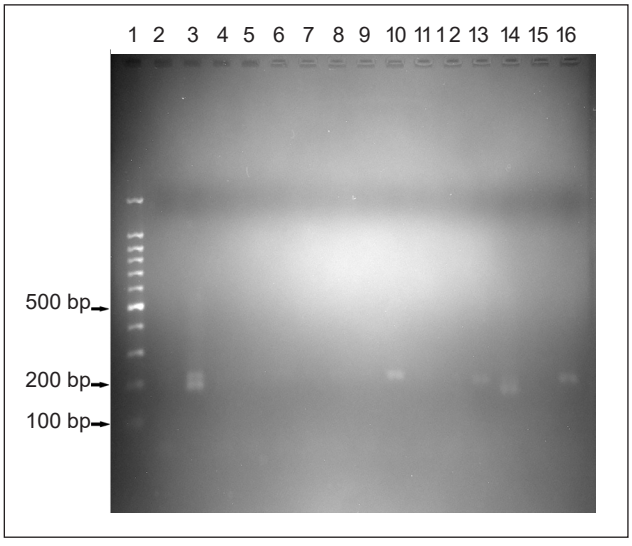

Figure 2. Multiplex polymerase chain reaction (PCR) agarose gel results indicating the detection of *Mycoplasma gallisepticum* and *M. synoviae* in some PCR products
Note. Lane 1 = 100 bp ladder; Lane 2 = Negative control; Lane 3 = Positive control; Lane 4-16 = Field samples

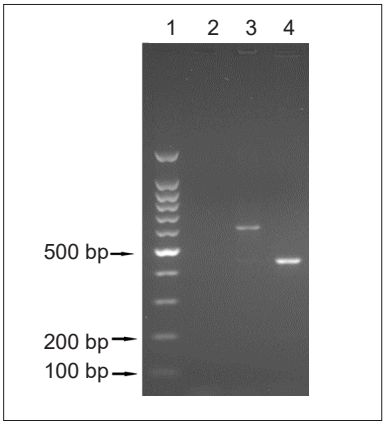

Figure 3. Gel electrophoresis result of the *Mycoplasma gallisepticum* isolates polymerase chain reaction product using *mgc2* strain-specific primers
Note. Lane 1 = 100 bp ladder; Lane 2 = Negative control; Lane 3 = Positive control; Lane 4 = Field isolate. The estimated product size for *the mgc2* gene of the field sample was 450 bp

Phylogenetic analysis of the partial sequence of this gene was done by constructing the neighbor-joining tree (Figure 4). According to this tree, the MG field isolate was found to be a progeny of a Thailand poultry strain (accession number: KX268616.1), although its *mgc2* pattern was not identical to other reference and international field isolates. Table 2 shows a list of isolates used to construct the phylogenetic analysis.

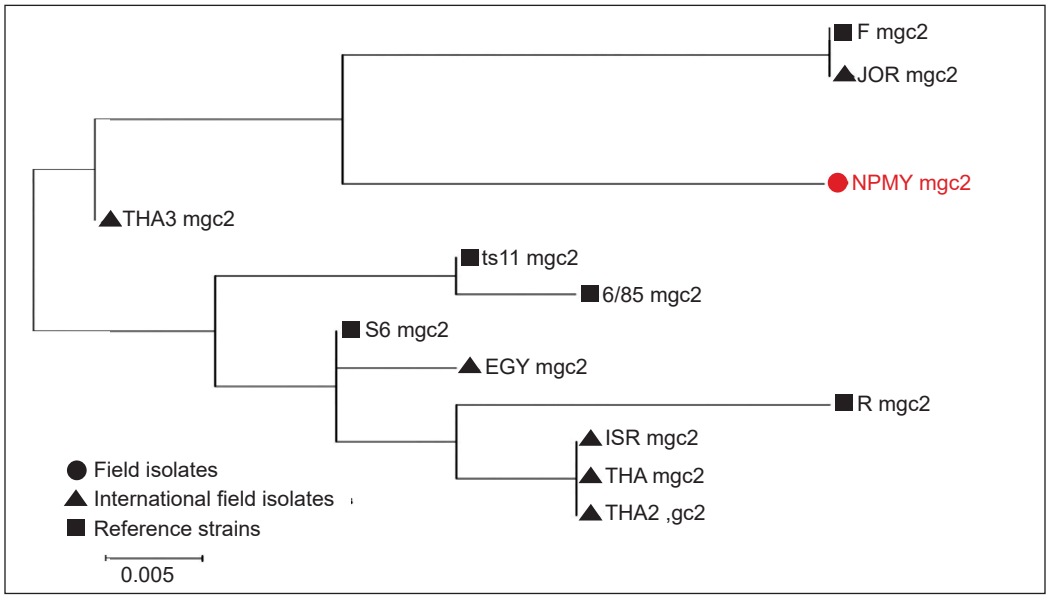

Figure 4. Phylogenetic tree of the *Mycoplasma gallisepticum* local strain (NPMY), international field isolates, and reference strains constructed based on *mgc2* partial gene sequence using the neighbor-joining algorithm. The country of origin of the international field isolates are shown as Egypt (EGY), Israel (ISR), Jordan (JOR), and Thailand (THA), respectively

Table 2
The isolates used in the phylogenetic analysis

Isolate name	Source	Country	Accession number
MG F	Reference strain		AY556230.1
MG JOR	Chicken	Jordan	HQ143379.1
MG NPMY	Jungle fowl	Malaysia	ON645899
MG THA	Chicken	Thailand	KX268618.1
MG THA2	Chicken	Thailand	KX268630.1
MG THA3	Chicken	Thailand	KX268617.1
MG ts-11	Reference strain		AY556232.1
MG 6/85	Reference strain		JQ770178.1
MG S6	Chicken	Thailand	KX268634
MG EGY	Chicken	Egypt	MW679029.1
MG R	Reference strain		AY556228.1
MG ISR	Chicken	Israel	HQ143377.1

Antimicrobial Minimum Inhibitory Concentration (MIC)

The antimicrobial MIC values of the MG PG31 reference strain were determined and compared to previous studies to optimize and validate the microdilution MIC protocol. Due to the failure to purify other isolate cultures, only three field isolates, including one MG isolate and two MS isolates, were successfully subjected to microdilution MIC. Results showed that all three isolates were resistant to erythromycin and chlortetracycline. The MIC values and the susceptibility profile of the isolates are shown in Table 3. The MG field isolates and one of the MS field isolates showed a multidrug-resistant (MDR) antimicrobial susceptibility profile.

DISCUSSION

This study aims to screen for the occurrence of MG and MS among various species of birds in Malaysia. Although the presence of MG among free-flying birds was detected by PCR in previous studies (Ganapathy et al., 2007), this study reported the isolation of MG and MS along with molecular characterization and determination of the antimicrobial susceptibility profile of the isolates. Most of the MG and MS isolates were collected from jungle fowl samples, indicating these microorganisms' affinity to fowl species. No MG was isolated from free-flying birds, although many free-flying culture samples showed mycoplasma colonies. It might be due to the overgrowth of commensal mycoplasmas inhibiting the growth of slow-growing MG (Bradbury, 2002; Ley, 2003). Isolation of MS from the free-flying birds' samples can indicate the significant role of free-flying birds in the circulation of avian mycoplasmosis. This finding is consistent with previous studies in which MS was detected in samples collected from pigeons (Benčina et al., 1987; Reece et al., 1986). Therefore, further studies investigating the phylogenetic analysis of MS isolated from free-flying birds are strongly suggested.

Molecular characterization of the field isolates indicated the presence of an MG field strain with a distinguished pattern of

Table 3
Antimicrobial minimum inhibitory concentration (MIC) values of Mycoplasma gallisepticum *(MG) and* M. synoviae (*MS) field isolates and reference strain*

Strain	MIC (μg/ml)								
	TIL	TIA	ENRO	DOX	ERY	LIN	CTET	TVN	TYLT
MG PG31	≤0.06	≤0.015	≤0.03	0.12	≤0.12	8	2	0.03	≤0.06
MG NPMY	≤0.06	0.06	2	0.5	64	0.5	8	0.03	0.12
MS NPMY1	≤0.06	0.25	0.25	0.25	64	2	8	0.12	0.25
MS NPMY2	32	1	32	0.5	64	4	8	0.25	4

Note. MG PG31 = Reference strain; MG NPMY = Local strain (isolated from aviary birds); MS NPMY1 = Local strain (isolated from free-flying birds); MS NPMY2 = Local strain (isolated from aviary birds); TIL = Tilmicosin; TIA = Tiamulin; ENRO = Enrofloxacin; DOX = Doxycycline; ERY = Erythromycin; LIN = Lincomycin; CTET = Chlortetracycline; TVN = Tylvalosin; TYLT = Tylosin

the *mgc2* gene. Phylogenetic analysis of this field strain showed that it is a progeny of a chicken MG field strain isolated in Thailand. This finding indicated the possible role of various bird species in the sustainability of avian mycoplasmosis by the occurrence of spillover infections between chicken and jungle fowl. It is consistent with a previous study indicating the strong possibility of spillover infection when the two species of birds belong to the same family (Dhondt et al., 2008; Farmer et al., 2005).

The antimicrobial susceptibility profile of the isolates was determined using microdilution MIC. The MIC values of the MG PG31 reference strain were compared to previous studies to validate the MIC assay. A consistent result was observed in the MIC value of the PG31 reference strain (Elbehiry et al., 2016; Gerchman et al., 2011, 2008; Khatoon et al., 2018; Li et al., 2010). Three field isolates, including one MG and two MS field isolates, were successfully subjected to the microdilution MIC assay. Failure to purify the isolates was the limit to success in determining the antimicrobial susceptibility profile of other isolates.

Considering the free-living nature of the study population and the MDR antimicrobial susceptibility profile of the isolates, these field strains may be transmitted from poultry, especially the MG isolated from jungle fowl. All the isolates showed resistance to erythromycin. This finding is consistent with previous studies that reported the resistance of MG field isolates to erythromycin (Bradbury et al., 1994; Taiyari et al., 2021; Tanner & Wu, 1992). The results of the microdilution MIC assay indicated resistance to enrofloxacin, lincomycin, chlortetracycline, and tylosin. These results agree with the increasing resistance of avian mycoplasmas against tetracyclines (Hannan et al., 1997), macrolides (Bradbury et al., 1994), and quinolones (Gerchman et al., 2011). However, tylosin, enrofloxacin, and tetracyclines are the most used and highly effective antimicrobial agents to control mycoplasmosis in poultry (Kleven, 2008). Enrofloxacin was less effective in treating MS infections in poultry (Stanley et al., 2001). In this study, one of the MS isolates was susceptible to enrofloxacin. It may be indicative of isolates that have not been transmitted from poultry.

CONCLUSION

This study investigated the role of non-poultry birds in the circulation of MG and MS in Malaysia. The culture and PCR results showed that MG and MS infections occur in the aviary and free-flying birds. Targeted gene sequencing of the isolates showed no identical pattern between the MG isolate and reference poultry strains used in this study. However, the phylogenetic analysis of the MG isolate showed that this isolate is a progeny of a poultry strain. The exposure of non-poultry birds to MDR, MG, and MS isolates might be another indication of the possible role of these birds in the circulation of disease. Unfortunately, this study could only evaluate 54 samples collected from different bird species in the Selangor state

of Malaysia. This caveat makes it difficult to draw a firm conclusion on this topic. However, results point out the possibility of MG and MS spillover infections. Therefore, constant surveillance is needed to identify MG and MS types isolated from non-poultry birds.

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