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Original Article

Genetic Variability and Antimicrobial Resistance Profile of Malaysian M. Gallisepticum and M. Synoviae Poultry Isolates

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ABSTRACT

M. gallisepticum (MG) and *M. synoviae* (MS) infections are among the most common and complicated respiratory diseases in birds. The infections can cause a huge loss of production performance in different types of poultry farming, including broilers and layers. Avian mycoplasmosis in Malaysia was detected many years ago, but there is a paucity of information on its genetic variability and antimicrobial susceptibility profile. Therefore, this study was carried out to isolate, molecular characterize, and determine the antimicrobial minimum inhibitory concentration of Malaysian MG and MS poultry isolates. A total of 492 choanal swab samples were collected from different poultry farms and subjected to isolation and PCR. Using immunofluorescence assay, 36.4% (179/492) MG and MS isolates were detected, out of which 26.8% (48/179) samples yielded MG colonies, and 73.2% (131/179) samples yielded MS colonies. Using PCR, a higher number of MG and MS were detected. *M. gallisepticum* was detected in 28% (138/492) of samples, while 61.2% (301/492) of samples were positive by PCR for MS. Phylogenetic analysis of the MG local isolates showed an identical pattern in both *pvpA* and *mgc2* genes with MG strain F. *M. synoviae* field isolates shared an identical pattern of *vlhA* gene with the MS strain MS-H. The isolates had the highest resistance to erythromycin, lincomycin, and chlortetracycline. The high number of positive MG and MS infections is suggestive of the continuous circulation of these pathogens among poultry in Malaysia. Therefore, continuous monitoring of the susceptibility profile of isolates to ensure effective treatment dosage is highly recommended.

INTRODUCTION

Mycoplasma gallisepticum (MG) is among the common causative agents of respiratory disease in birds in Malaysia (Zahraa *et al*., 2011; Taiyari *et al*., 2023). *M. gallisepticum* can cause considerable economic losses to poultry industries via reduction in production performance of infected chickens (Burnham *et al*., 2003; Parker *et al*., 2003; Winner *et al*., 2003). Like MG, *Mycoplasma synoviae* (MS) infection could also cause huge economic losses to the production level of poultry industries (Ferguson-noel & Noormohammadi, 2013). *M. synoviae* can spread faster than MG, and it can cause synovitis, in addition to respiratory disease (Ferguson-noel & Noormohammadi, 2013). Chickens infected by MG and MS suffer from respiratory problems that lead to reduced egg production and weight gain (Marouf *et al*., 2020). In addition, MG and MS are vertically transmitted pathogens that can cause embryonic mortality and reduction in hatchability of eggs (Bencina *et al*., 1988a, 1988b; Raviv & Ley, 2013).

The detection of MG and MS infections is done via culture, serological and molecular techniques. The culture technique includes the isolation and subsequent identification 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). Polymerase chain reaction (PCR) offers a rapid and convenient diagnosis of pathogenic mycoplasmas. However, a few studies reported false diagnosis of MG by PCR (Kempf *et al*., 1997; Ganapathy & Bradbury, 1999). 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 isolates (Ferguson *et al*., 2005). In Malaysia, studies have reported a high occurrence of mycoplasmosis in poultry farms (Zahraa *et al*., 2011). Previous phylogenetic analysis of Malaysian MG field isolates were identical to F and S6 strains based on pMGA and *pvpA* partial gene sequencing (Yasmin *et al*., 2018).

Despite all the efforts to control MG infection in Malaysia, previous studies have reported its high occurrence in poultry farms (Yasmin *et al*., 2014). Antimicrobial medication is one of the main control strategies for poultry mycoplasmosis (Kleven, 2008). Mycoplasmas do not have a cell wall, and are therefore resistant to β-lactam antibiotics such as penicillins or cephalosporins. Macrolides, tetracyclines, and fluoroquinolones are considered to be effective to treat avian mycoplasmosis (Kleven, 2008). *In vitro* determination of the antimicrobial susceptibility profile of avian mycoplasmas was described by Hannan (2000). Although mycoplasmas tend to be susceptible to macrolides, tetracyclines, and fluoroquinolones, there is a growing trend of resistance development against enrofloxacin, tylosin, erythromycin, and oxytetracycline (Taiyari *et al*., 2021). Unfortunately, there is a paucity of data on the antimicrobial susceptibility profile of Malaysian MG and MS isolates. Therefore the aim of this study is to isolate, molecular characterize, and determine the antimicrobial susceptibility profile of Malaysian MG and MS field isolates.

MATERIALS AND METHODS

Sample Collection

A total of 492 choanal slit samples were collected from six broiler breeder farms and six layer farms. These farms were different in herd size and were located in five different states of Peninsular Malaysia. To control MG infection, Live F strain vaccine were used in these farms. No MS vaccination protocol were used in these farms. Samples were collected from chickens with clinical signs related to avian mycoplasmosis. All of the sampling procedures and the number of samples were approved by the Institutional Animal Care and Use Committee (IACUC) (UPM/IACUC/AUP-R069/2019). For identification of the isolates and further characterization, *M. gallisepticum* PG31 reference strain and *M. synoviae* WVU 1853 were obtained from ATCC.

Isolation and Identification

The isolation protocol and preparation of the type of medium used for isolation were adapted from Ferguson-noel & Kleven (2016). Frey medium with 15% swine serum (FMS) was used to isolate MG and MS. After swabbing the choanal slit, swab samples were inoculated into the FMS broth medium. Once broth medium changed color during incubation, samples were subjected to agar inoculation. Plates with colonies were assayed using a stereomicroscope. Tiny, smooth colonies of 0.1–0.5 mm diameter with dense, elevated centers with a "fried egg appearance" were considered mycoplasmal colonies. The identification of MG and MS colonies was respectively made using direct and indirect immunofluorescence assay (IFA), according to Ferguson-noel & Kleven (2016). The direct immunofluorescence technique was used to identify MG by using a high-titer MG specific rabbit polyclonal antibody (IgG) conjugated with Alexa fluor 488 (Bioss Antibodies, USA). The indirect immunofluorescence technique was used for the identification of MS. The MS-specific chicken polyclonal antibody (IgY) was used as the primary antibody. For the secondary antibody, goat anti-chicken IgY conjugated with Phycoerythrin (PE) was used (Abcam, UK). After IFA staining, the agar blocks were viewed using a fluorescence microscope (Figure 1).

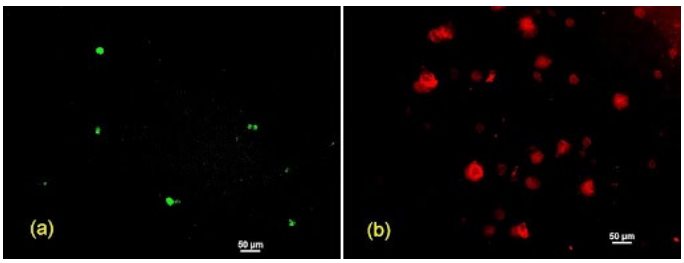

Figure 1 – Immunofluorescence (IF) staining of MG and MS colonies. (a) Alexa fluor 488 stained the MG colonies bright green (apple green); (b) Phycoerythrin (PE) was used for MS colonies and stained the colonies red.

Molecular Detection and Characterization

In addition to isolation, samples were also tested for MG and MS using multiplex species-specific PCR targeting 16S rRNA (Moscoso *et al*., 2004). Two sets of primers targetting 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 for molecular identification. Extraction of DNA was done using the QIAGEN extraction kit (QIAamp DNA Mini Kit). The reaction solution was prepared at 25µl. Initial denaturation was at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. A final extension was performed at 72°C for 5 min.

For molecular characterization, positive cultures of MG and MS were subjected to strain-specific PCR assays. Strain-specific PCR assays were designed to target single genomic loci of virulence genes. For MG, *mgc2* and *pvpA* cytadhesin genes were amplified in order to molecular characterize the isolates. For MS, the *vlhA* hemagglutinin gene was amplified for molecular characterization. Fifteen MG and twenty MS isolates were subjected to a strain-specific PCR assay. The sequences of primers used for strain-specific PCR are shown in Table 1. For the *mgc2* gene, initial denaturation was at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 60 sec, and final extension was performed at 72°C for 5 min. For the *pvpA* gene, initial denaturation was at 94°C for 5 min, followed by 35 cycles of 94°C for 60 sec, 55°C for 60 sec, and 72°C for 90 sec, and final extension was performed at 72°C for 5 min. For the *vlhA* gene, initial denaturation was at 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec, and final extension was performed at 72°C for 15 min. PCR products were purified and then sequenced using Sanger sequencing. Sequences of virulent genes were first aligned and then subjected to phylogenetic analysis using the MEGA 7 software.

Table 1 – Strain-specific primers used in the PCR assay.

Target gene	Primers sequence	Amplicon size
mgc2	F: CGCAATTTGGTCCTAATCCCCAACA R: TAAACCCACCTCCAGCTTTATTTCC	300 bp-860 bp
pvpA	F: GCCAMTCCAACTCAACAAGCTGA R: GGACGTSGTCCTGGCTGGTTAGC	430 bp-660 bp
vlhA	F: GAT GCG TAA AAT AAA AGG AT R: GCT TCT GTT GTA GTT GCT TC	316 bp-394 bp

Antimicrobial Susceptibility Profile

For the determination of antimicrobial susceptibility profile of MG and MS isolates, microdilution minimum inhibitory concentration (MIC) assay was employed, according to Hannan (2000). Customized sensititer plates were obtained from Thermo Fischer (CMP1VEAH). Briefly, after the preparation of pure cultures using inoculation techniques and immunofluorescence staining, stock pure cultures were preserved by adding 5% of v/v sterile glycerol. Stock cultures were then used to perform viable counting. Once MG or MS field isolates reached 10^4 color changing units (ccu) in the viable counting stage, microdilution MIC plates were inoculated. To determine the MIC value of the field isolates, the plates were monitored three times per day for up to 72 hours for the initial MIC value. For the validation of the MIC test, the MG PG31 ATCC reference strain was subjected to MIC assay, and its MIC values were compared with previous studies.

RESULTS

Isolation and Identification

Both isolation and PCR techniques were used to detect MG and MS. A total of 179 MG and MS were isolated from 492 samples. The result of isolation and PCR detection is shown in Table 2. Using the immunofluorescence assay, 48 isolates were identified as MG isolates, and 131 were identified as MS isolates. 11 samples had both MG and MS isolates. Using PCR, MG and MS were detected in 27.6% (136/492) and 61.2% (301/492) of samples, respectively. Twenty-six samples were positive for both MG and MS by PCR.

Table 2 – Isolation and PCR assay result of the samples.

Farm ID	Nº Samples	MG isolates (%)	MG PCR (%)	MS isolates (%)	MS PCR (%)
A[BB]	45	10 (22%)	25 (56%)	38 (84%)	41 (91%)
B[BB]	75	0	5 (7%)	0	56 (75%)
C[BB]	75	0	2 (3%)	0	24 (32%)
D[BB]	45	0	18 (40%)	29 (64%)	38 (84%)
E[BB]	60	20 (33%)	33 (55%)	0	8 (13%)
F[BB]	30	0	0	13 (43%)	30 (100%)
G[L]	10	2 (20%)	5 (50%)	6 (60%)	8 (80%)
H[L]	45	7 (16%)	18 (40%)	19 (42%)	32 (71%)
I[L]	40	5 (13%)	16 (40%)	17 (43%)	25 (63%)
J[L]	13	0	2 (15%)	0	6 (46%)
K[L]	24	4 (17%)	14 (58%)	5 (21%)	16 (67%)
L[L]	30	0	0	4 (13%)	17 (57%)
Total	**492**	**48**	**136**	**131**	**301**

[BB] broiler breeder; [L] layer

Genetic Variability of Isolates

The sequences of strain-specific PCR products were further analyzed by constructing the phylogenetic tree. The phylogenetic trees of MG and MS field isolates were constructed based on the *mgc2*, *pvpA*, and *vlhA* genes (GenBank accession numbers: OP957010, OP957011, OP957012, OP957013, OP957014, OP957015, OP957016, PP277658 and PP277659) (Figure 2, Figure 3, and Figure 4). Figure 2 portrays the identical pattern of the MG isolates to the reference F strain based on the *mgc2* gene. According to the gene-targeted sequencing of the *pvpA* gene (Figure 3), MG isolates were identical to the reference F strain. Phylogenetic analysis of the MS isolates based on the *vlhA* gene showed an identical pattern between the field isolates and the MS-H reference strain (Figure 4).

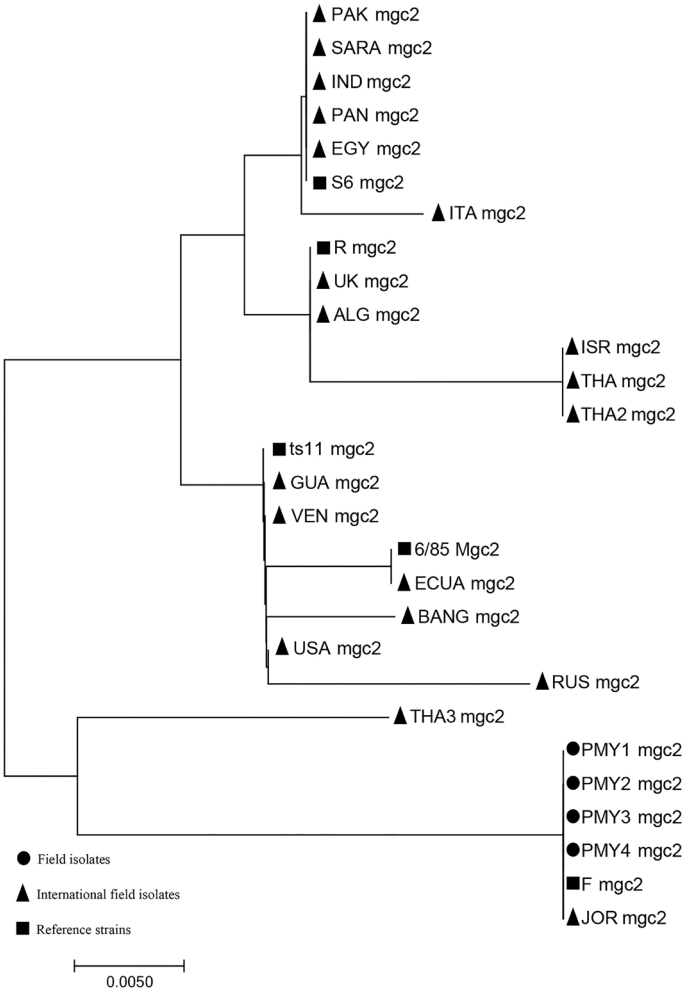

Figure 2 – Phylogenetic tree of MG field isolates based on the *mgc2* gene using neighbor joining algorithm (rooted). PAK: Pakistan; SARA: Saudi Arabia; IND: India; PAN: Panama; EGY: Egypt; ITA: Italy; UK: United Kingdom; ALG: Algeria; ISR: Israel; THA: Thailand; GUA: Guatemala; VEN: Venezuela; ECUA: Ecuador; BANG: Bangladesh; USA: United States of America; RUS: Russia; JOR: Jordan.

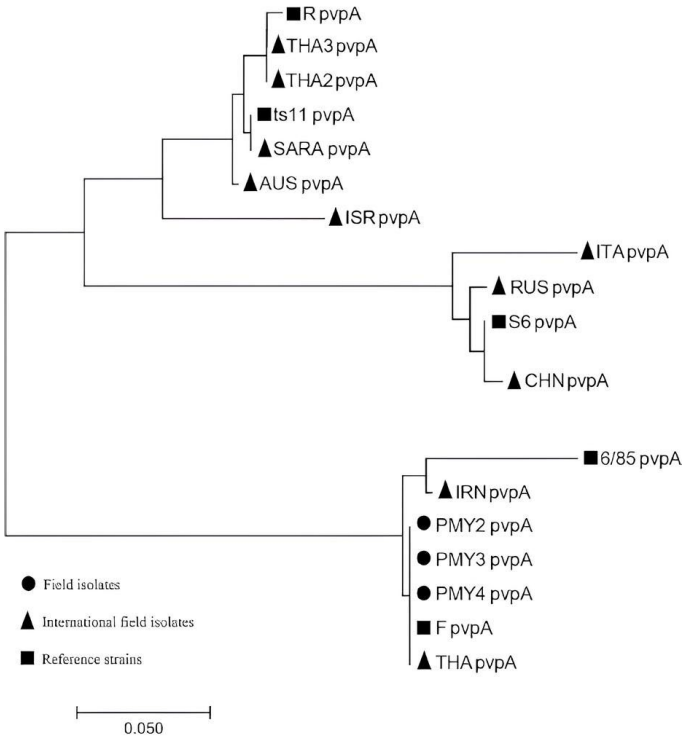

Figure 3 – Phylogenetic tree of MG field isolates based on the *pvpA* gene using neighbor joining algorithm (rooted). THA: Thailand; SARA: Saudi Arabia; AUS: Australia; ISR: Israel; ITA: Italy; RUS: Russia; CHN: China; IRN: Iran.

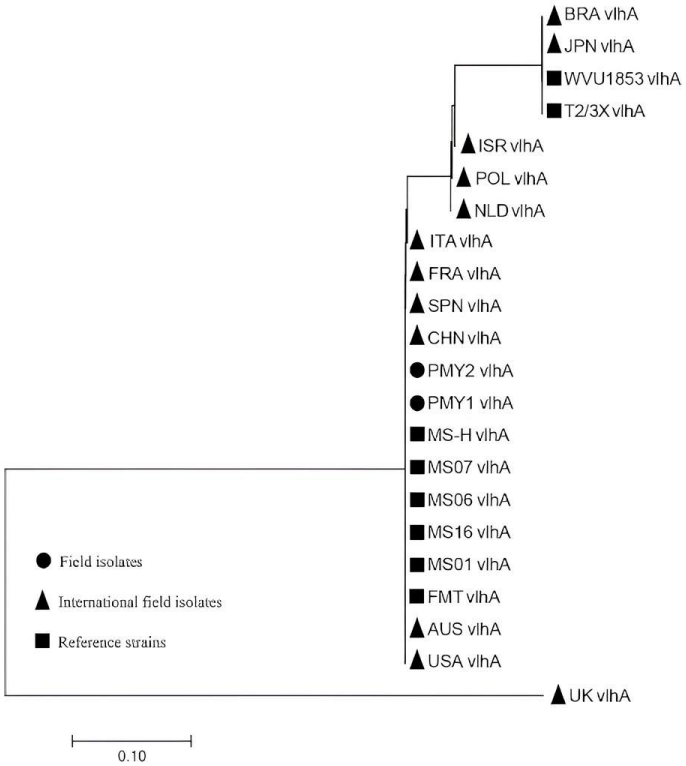

Figure 4 – Phylogenetic tree of MS field isolates based on the *vlhA* gene using neighbor joining algorithm (rooted). BRA: Brazil; JPN: Japan; ISR: Israel; POL: Poland; NLD: Netherlands; ITA: Italy; FRA: France; SPN: Spain; CHN: China; AUS: Australia; USA: United States of America.

Antimicrobial Susceptibility

Antimicrobial susceptibility profile of the isolates was determined by comparing their microdilution MIC values (Table 3) with clinical breakpoints or cut-off points. Four MG and 2 MS isolates were subjected to antimicrobial minimum inhibitory concentration assay.

Table 3 – MIC values of antibiotics against MG and MS isolates.

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 PMY1	≤0.06	0.06	2	0.5	64	0.5	8	0.03	0.12
MG PMY2	≤0.06	≤0.015	0.12	0.5	0.5	2	1	≤0.015	≤0.06
MG PMY3	≤0.06	≤0.015	0.12	0.25	0.5	2	4	≤0.015	≤0.06
MG PMY4	≤0.06	≤0.015	0.12	0.25	≤0.012	4	4	≤0.015	≤0.06
MS PMY1	≤0.06	0.12	0.25	≤0.06	64	0.5	4	0.03	≤0.06
MS PMY2	≤0.06	0.25	0.25	0.25	64	2	8	0.12	0.25

TIL: Tilmicosin; TIA: Tiamulin; ENRO: Enrofloxacin; DOX: Doxycycline; ERY: Erythromycin; LIN: Lincomycin; CTET: Chlortetracycline; TVN: Tylvalosin; TYLT: Tylosin

DISCUSSION

A higher number of positives were detected for both MG and MS using PCR assay in comparison to the isolation technique (437 vs 179). This indicates the higher sensitivity of PCR in the detection of MG and MS from field samples (Amores *et al*., 2010). Similarly, experimental studies designed to compare the diagnostic techniques for avian mycoplasmosis showed significant differences between PCR and culture in diagnosing MG and MS (Salisch *et al*., 1998; Feberwee *et al*., 2005). Polymerase chain reaction assay was found to be fast, cheap, and sensitive in the detection of MG and MS (Feberwee *et al*., 2005). However, PCR results are not reliable in detecting atypical MG strain infections or *Mycoplasma imitans* infections (Kempf *et al*., 1997; Ganapathy & Bradbury, 1999). In this study, PCR could not differentiate active infections from inactive infections. This finding was observed in samples collected from a recently treated broiler breeder farm, where there were no samples detected as positive for MG and MS by the culture technique. However, numerous samples were positive for MG and MS via PCR (data not shown). Therefore, in a field assessment, culture should always accompany PCR assay to prevent such problems.

In this study, a higher number of MS was detected using both culture and PCR techniques compared to MG. In general, it is believed that MS can spread faster than MG, making it more prevalent (Olson & Kerr, 1967), as the outcomes of this study show. However, previous studies in Malaysia detected more MG than MS (Tan *et al*., 2005; Ahmad, 2012). In the study conducted by Tan *et al*. (2005), 11% of the samples were positive for MG by culture, and 3% were positive for MS. Similarly, in the study conducted by Ahmad (2012), MG was detected more than MS using both culture (9% vs. 2%) and PCR (24% vs. 5.6%). The increase in the number of MS infections in recent years could be due to several factors. The wide use of vaccination and antimicrobial treatment against MG could be one of the possible factors, paving the way for the faster spread of MS. Another factor that could lead to an increase in MS number is the organism's long-term survival in environmental samples such as litter (Marois *et al*., 2000, 2005). This factor is highly associated with the management system of the farm. In other words, the reuse of litter could increase the risk of MS infection. The isolation of both MG and MS from a sample was found more often in layer chicken samples than in broiler breeder samples, which might be explained by the higher stocking density in layer farms.

To further characterize the isolates, virulent genes of both MG and MS were subjected to sequencing. These genes were inclusive of *mgc2* and *pvpA* for MG, and the *vlhA* gene for MS, which have been used to characterize MG and MS isolates (Boguslavsky *et al*., 2000; Liu *et al*., 2001; Garcia *et al*., 2005; El-Gazzar *et al*., 2012; Yasmin, 2013). Different strains of MG and MS have varied gene sizes, and only a single copy of this gene can be found in the bacterial genome. This study showed that four of the field MG isolates were grouped in the same cluster of the vaccine F strain. The findings of this study are similar to another study that reported the close relationship of MG positive samples with the MG F strain (Zahraa *et al*., 2011; Yasmin, 2013). According to the gene-targeted sequencing of

mgc2, all isolates were 100% identical. However, upon targeting the *pvpA* gene, one of the poultry isolates was slightly different from the others. This difference could be due to the more polymorphic nature of the *pvpA* gene (Jiang *et al*., 2009). Although *mgc2* is one of the recommended genes for MG molecular detection, some MG isolates could not produce bands in the gel electrophoresis of *mgc2* in this study. Similarly, some MG isolates could not be characterized based on the *pvpA* gene in this study. The *pvpA* gene has been used to characterize MG isolates (Liu *et al*., 2001). These studies, however, amplified the gene using nested PCR. For MS, two isolates were successfully sequenced based on the *vlhA* gene. Both isolates had an identical pattern to the MS-H reference strain. Many MS isolates did not show any band in the gel electrophoresis of the *vlhA* PCR product. The *vlhA* gene is the most commonly used gene for targeted sequence typing of MS strains. However, some factors can cause failure in the targeted sequence typing of MS isolates based on the *vlhA* gene. These factors include the length of the amplicon, level of homogeneity, the possibility of multiple bands, and virulent strains (May & Brown, 2011; El-Gazzar *et al*., 2012).

The MIC values of MG PG31 were similar to Hannan (2000), indicating the validity of the MIC tests. In this study, tylvalosin was found to be the most effective antibiotic to treat avian mycoplasmosis. Similar findings have been observed in Thailand, Egypt, Iran, and Europe (Pakpinyo & Sasipreeyajan, 2007; Behbahan *et al*., 2008; Kreizinger *et al*., 2017; El-Hamid *et al*., 2019). All of the isolates tested for their antimicrobial susceptibility profiles were susceptible to tiamulin. 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*., 2017). A high number of isolates were resistant to macrolides (erythromycin and lincomycin). Similar findings have been observed in Egypt and Iran (Behbahan *et al*., 2008; El-Hamid *et al*., 2019). The fast development of resistance against macrolides has been reported by Gautier-Bouchardon *et al*. (2002), suggesting a mechanism of natural resistance, as already observed in *Mycoplasma hominis* (Furneri *et al*., 2000). Twenty percent of isolates in this study were resistant to enrofloxacin. Although it has been found that there is a quinolone resistance determining region in the genome of mycoplasmas, it has been found that MG develops resistance towards enrofloxacin gradually and slower in comparison to macrolides (Gautier-Bouchardon *et al*., 2002). Among tetracyclines, chlortetracycline had the highest number of resistant isolates, which is consistent with studies from Thailand and Egypt (Pakpinyo & Sasipreeyajan, 2007; El-Hamid *et al*., 2019). Chlortetracycline is believed to be used more frequently, and has been introduced for longer periods (Pakpinyo & Sasipreeyajan, 2007). Studies investigating the mutation in MG isolates have shown that MG can develop resistance against antimicrobial agents, especially macrolides and quinolones (Wu *et al*., 2005; Li *et al*., 2010; Lysnyansky *et al*., 2012; Taiyari *et al*., 2021). This study also reported a similar outcome, whereby the highest number of resistant isolates was related to erythromycin.

CONCLUSION

This study shows that MG and MS infections are still a problem among poultry in Malaysia. A higher occurrence of MS was observed in this study compared to previous studies conducted in the country. Phylogenetic analysis of the isolates indicated that MG isolates are in the same cluster of the MG F strain, and MS isolates are in the same cluster of the MS MS-H strain. Differences in the MIC values of isolates in the same cluster indicate the lack of an optimized treatment regime. The detection of AMR isolates highlights the need for a prudent use of antibiotics in Malaysia, and for antimicrobial sensitivity tests prior to the treatment of any infections.

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AUTHOR CONTRIBUTIONS

Conceptualization, HT, ZZ, JA and NMF; methodology, HT and ZZ; software, HT; validation, ZZ, JA and NMF; formal analysis, HT; investigation, HT; resources, ZZ, JA and NMF; data curation, HT, ZZ, JA and NMF; writing—original draft preparation, HT; writing—review and editing, HT; visualization, HT; supervision, ZZ, JA and NMF; project administration, HT, ZZ, JA and NMF; funding acquisition, ZZ and NMF. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

Data will be available upon request.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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