Original Article

Detection of *Mycoplasma gallisepticum*, Isolation, and Determination of Tylosin Susceptibility of Isolates from Commercial Chickens

Kachabi, K[1], Pourbakhsh, S. A[1*], Zahraei Salehi, T[1]

1. *Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

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Corresponding Author's E-Mail:
poursaba@yahoo.com

ABSTRACT

Mycoplasma gallisepticum (MG) is a contagious avian pathogen that causes financial losses to the poultry industry. Isolation of the pathogen is difficult and time-consuming, and therefore, far from a routine method. Serological testing methods to detect antibodies resistant to MG are widely used in routine diagnosis. Tylosin is a class of macrolide antibiotics tremendously administered in veterinary medicine for the treatment of mycoplasmosis and prophylaxis. This study aimed to detect MG by immunoassay testing, culture, and polymerase chain reaction (PCR) in commercial poultry farms and to investigate the tylosin susceptibility of the isolates. To verify the presence of antibodies resistant to MG, 750 blood samples were randomly collected from 38 broiler farms from 2019 to 2022 in Mazandaran and Golestan provinces, Iran, and rapid slide agglutination (RSA) assay was performed. Positive results were analyzed by the enzyme-linked immunosorbent assay (ELISA) for further investigation. Here, 920 swab samples were collected from 38 non-vaccinated commercial farms for culture, and PCR tests were performed for the isolated strains. The activities of tylosin were tested *in vitro* against these isolates using the broth microdilution method. The lowest antibiotic concentration that resulted in a color change was considered the minimum inhibitory concentration (MIC) value. Twenty-four (63.1%) farms were positive in the RSA test, and 21 (55.2%) farms were positive in the ELISA test. Nine (23.68%) of the farms grew on culture media, and 8 (21.05%) were detected as Gallisepticum species by PCR. The geometric mean of MIC for tylosin was 5.75 µg/ml, MIC50 was 4 µg/ml, and MIC90 was 8 µg/ml. The results indicated that commercial farms were infected with MG. Considering the ability of MG to spread and the probable use of the RSA test as a rapid and cheap method, it can be argued that ELISA and RSA serological tests can be used to find MG in poultry flocks, and the positive result should be confirmed by standard microbiological tests or PCR. It was also found that the isolated parts of MG changed their sensitivity to tylosin, indicating the need for routine testing to optimize treatment dose and efficiency.

Keywords: *Mycoplasma gallisepticum*, MIC, Tylosin, ELISA

1. Introduction

Considerable attention has been devoted to the adverse effects of *Mycoplasma gallisepticum* (MG) in the poultry industry Yadav, Singh (1). A bacterium in the Mollicutes class and the *Mycoplasmataceae* family known as MG can cause chronic respiratory disease (CRD) in chickens of all ages. This bacterium acts like influenza in humans and makes birds noticeably ill. Common symptoms include watery eyes, rales in the tracheas, nasal discharge, and cough, which cause chickens to have a decreased appetite and eventually lose weight. The disease can also affect pheasants and quail, which show respiratory sounds, cough, and nasal discharge. It causes economic losses through increased mortality and decreased feed intake and egg production efficiency. The pathogen causes an increase in drug costs and a reduction in the commercial profitability of poultry production worldwide. Clinical manifestations can characterize MG in numerous ways; however, overt clinical signs do not always precede significant economic loss. The most prominent symptom of MG is CRD in broiler breeds, although it may result from various etiologic agents in a multifactorial disease state (2).

In recent years, the predominant methods for detecting MG have been based on culture, serology, or nucleic acid amplification. The best way to diagnose MG is to test it explicitly for cell culture contamination. In 2020, the effect of various types of cotton swabs stored at different temperatures was investigated on the detection of MG and *Mycoplasma synoviae* (MS) (3). The study combined conventional culture analysis with modern molecular detection methods. In a major advance in 2023, improved loop-mediated isothermal amplification (LAMP) was integrated with a TaqMan probe (LAMP-Taqman method) for the detection of MG (4). The authors performed parallel experiments on 120 samples from a pair of chickens and obtained accuracy, sensitivity, and specificity of 97.5%, 100%, and 96.3%, respectively, for the proposed method. Accordingly, the issue of the detection of MG has always been a controversial topic in related work.

Serologic methods help monitor flocks as part of MG control programs aimed to diagnose infected birds. A positive serological test and clinical signs or a history of MG disease allow a more reliable diagnosis of possible infection and detection of pathogenic infestation. Enzyme-linked immunosorbent assay (ELISA) is a standard method employed for such cases (5). In the acute stage, severe infection is characterized by a significant increase in the MG population in the upper respiratory tract. As a result, the infection becomes highly disseminated among the animals in the herd. To optimize isolation efficiency, we need to collect samples from flocks to culture MG before antimicrobial therapy (6). For *in vitro* culture of MG, it is necessary to inoculate suspensions of air sac, lung, nasal concha, or sinus fluid exudates directly onto agar medium or mycoplasma broth (7).

There are various solutions to eliminate the effect of MG, including antibiotics, such as erythromycin, tylosin, and tetracyclines. Clinical symptoms take time to appear because they develop slowly; therefore, the infection may remain unknown for some time. Antibiotic treatments can successfully reduce clinical symptoms and suppress the transmission of the disease. Although they probably prevent poultry mycoplasmosis from causing economic losses, they are an effective treatment to minimize mortality. Nevertheless, such preventive measures should preferably be implemented in a short time because isolates can develop antimicrobial resistance (AMR) to antimicrobial agents. Reports indicate that MG can be effectively controlled by numerous antimicrobial agents, such as gentamicin, lincomycin, spectinomycin, tetracyclines, macrolides, tiamulin, fluoroquinolones, streptomycin, salinomycin, and spiramycin (8). The ability of MG to alter its surface proteins enables the pathogen to reduce the efficacy of antimicrobial agents, particularly those that target surface proteins (9).

Strains of MG vary widely in terms of their biological properties, pathogenicity, infectivity, tissue tropism, and transmissibility. Moreover, their antigens differ considerably among strains and cause phenotypic

variation, which partly explains the chronic infection of MG despite a strong immune system (2). A feasible approach to monitor the antimicrobial susceptibility of pathogenic isolates is to avoid AMR. Therefore, several tests are available to investigate the antimicrobial susceptibility profile of isolated and field-collected pathogens. The minimum inhibitory concentration (MIC) test in modified broth microdilution can be used to reliably determine the antimicrobial susceptibility profile of MG field isolates (7). In MG-exposed layers of multi-age complexes, the addition of very low doses of tylosin to poultry feed can mitigate the adverse effects of MG on egg production (2). Nonetheless, MG can develop various resistance levels to tylosin when subinhibitory concentrations are used against the pathogen (6).

Iranian primary and multiplier breeders of chickens and hatcheries typically employ a variety of mycoplasma management strategies. These preventive strategies are necessary to ensure that many layer and broiler flocks are not infected by the organisms during production periods. Although breeding flocks often have MG infections, line and parent flocks are mycoplasma-free. However, live mycoplasma vaccines are not widely licensed in the country (10), and the control of mycoplasma infections by vaccination is only moderately effective. Several studies have been conducted to identify and isolate MG in Iran. Shahriari, Afsharifar (11) attempted to detect and isolate MG in chicken farms using restriction fragment length polymorphism and polymerase chain reaction (PCR) assays and culture methods in Fars province, Iran. Hosseini, Bozorg (12) studied the isolates of MG in several geographical areas of Iran using random amplification of polymorphic DNA. Tekkalan (13) discovered MG among commercial poultry in Khouzestan province, Iran. In a population of broiler flocks in northern Iran, antibiotics are used to control mycoplasmas in addition to biosecurity measures (14).

Despite this interest, there are still many gaps and drawbacks that need to be addressed immediately. The trails of MG require careful testing to determine the tylosin susceptibility of isolates from commercial chicken flocks. The concept of PCR and reporting MIC50 and MIC90 values play a key role in determining drug levels. The question that arises here is how the proposed method can detect MG infection. The novelty here is the accuracy and reliability of the proposed method, which has not yet been considered in Iran. This study aimed to isolate and identify MG in industrial poultry farms using rapid slide agglutination (RSA), ELISA, and culture methods, and then confirm *Gallisepticum* species by PCR test. In the following, the sensitivity of the isolates to the antibiotic tylosin is verified.

2. Materials and Methods

2.1. Sampling

Having obtained ethical approval, 750 blood samples were randomly collected from 38 broiler breeder farms from Mazandaran and Golestan provinces (Iran) with respiratory signs for serological tests, and ELISA and RSA tests were used to check antibodies and MG. A total of 920 swab samples were collected from the 38 flocks, and the mycoplasma broths were directly inoculated with isolates from tracheal swabs, choanal clefts, and nasal swabs. The inoculated broths were stored in cold packs and processed overnight but for no longer than 24 hours. For each culture medium, 3 to 5 swabs were pooled to increase the probability of isolation (7). We inoculated Frey agar and Frey broth with the smear and then centrifuged 1 ml of the Frey broth culture at 12,000 g for 5 min. Additional information on the samples included in the work is summarized in table 1.

Table 1. The supplementary dataset used for the sampling

Sterile water	Temperature of incubation	Storage temperature	Suspension used for PCR
1µl of the suspension	37 Celsius degrees	-20 Celsius degrees	1µl

2.2. Rapid Slide Agglutination

Briefly, 50 µl of fresh serum was mixed with 50 µl of antigen (SOLEIL Diagnostics, France) and incubated at room temperature for 2 min before reading the results. The samples positive in the rapid test were examined by ELISA assay for further investigation.

2.3. ELISA

ELISA test kits (IDEXX, Synbiotics, Biochek) were used to perform the test according to the manufacturer's instructions. Plates were then tested using an ELISA reader to determine the optical density of the positive, negative, and control samples. The readings were interpreted according to the manufacturer's guidelines. In the ELISA procedure, a polystyrene plate with a solid surface and affinity for bacteria, other antibodies, and hormones is combined with an antibody. After adding the antigen-antibody mixture to the antigen-coated microtiter, the free antibodies were removed by washing. A second specific antibody with an enzyme was added to the primary antibody. The plate was washed to remove the free enzyme-bound secondary antibodies, and finally, the substrate was added.

2.4. Culture

The swabs were propagated in Frey's culture media. The composition of the culture medium consisted of a broth-based mycoplasma medium (22.5 g), horse serum (120 ml), glucose (3 g), yeast (35 ml), cystine hydrochloride (0.1 g), nicotinamide adenine dinucleotide (0.1 g), thallium acetate 10% (3 ml), phenol red 1% (2.5 ml), penicillin G (10,000 units per ml), and water (up to one liter). Finally, the pH of the environment was adjusted to 7.8 with sodium hydroxide (20%). Each isolate was cultured therein and stored at 37°C under microaerophilic conditions (2% carbon dioxide). These conditions were maintained until the phenol-red indicator turned yellow. If the color did not change, another passage was performed after 7-10 days, on broth (1:10 v/v ratio) and agar media. Broth and agar culture media were checked twice daily until the color of the media turned yellow. One colony was identified under the microscope on the agar culture medium. The appearance of fried-egg-shaped colonies under the dissecting microscope indicated a positive result (2).

2.5. Polymerase Chain Reaction

To confirm the presence of MG, we used PCR. Accordingly, DNA extraction was performed using the High Pure PCR Template Preparation Kit (Roche). When performing PCR, 1 ml of Frey's broth culture was centrifuged at 12,000x for 5 min. The supernatant was then removed, and the pellets were suspended in 50 µl of sterile water. An equal ratio (1~1) of suspension was then used, and 50 µl of the suspension was utilized to perform PCR. Each mixture contained 5 µl of 10X PCR buffer (500 mM KCl). The procedure involved the addition of 200 mM Tris-hydrochloride, pH 8.41, 1.5~1 of 10 mM of each nucleotide (dCTP, dATP, dGTP, and dTTP), 2.5µl of each of the general mycoplasma primers (60 ng/µl), and 0.2 µl of *Taq* DNA polymerase, 1 µl of each of the MG specific primers, and 1 µl of the broth culture of mycoplasmas. Reaction mixtures were adjusted to a total volume by adding distilled water. Primers used in this study: forward primer: S-AACACCAGAGGCGAAGGCGAGG-3', reverse primer: S-ACGGATTTGCAACTGTTTGTATTGG-3'. Notably, MG was expected to have an amplicon size of 531bp (15). The sequences of primers GPO-3 and MGSO were S-GGGAGCAAACAGGATTATATATACCCT -3' and 5'-TGCACCATCTGTCACTCTGTTAACCTC-3', respectively. A species-specific primer pair was also used to detect MG, with forward and reverse primer sequences (S-AACACCAGAGGCGAAGGCGAGG3' and S-ACGGATTTGCAACTGTTTGTATTGG3, respectively). Complementarity of the primer sequences to the available sequence data of the 16S rRNA gene of the four avian mycoplasmas was determined using the sequence analysis software package Primer Designer, version 2.0 (Scientific and Educational Software, ser. no. 50178. PO Box 440. State Line, PA 17263, USA). To perform DNA amplifications, the samples were heated to 94°C for 5

min followed by 35 cycles of three durations and temperatures (94°C for 30 s, 55°C for 30 s, and 72°C for 30 s). These corresponded to primer extension, primer annealing, and target DNA denaturation. The last step of the extension cycle was conducted at 72°C for 10 min. To detect the PCR products, the amplified DNA samples were analyzed by electrophoresis on 2.0% agarose gels in TAE buffer (40 mM tris acetate, 1 mM EDTA). The sample solution contained 0.5 µg/ml ethidium bromide. Finally, gel electrophoresis was performed at 110 V for 45 min. An ultra-violet transillumination (at 302 nm) on Polaroid 667 films was used to photograph the gels, along with a 100 bp DNA ladder as a molecular weight standard (15).

2.6. Minimum Inhibitory Concentration

The broth microdilution method (Tanner and Wu method) with PPLO broth medium (pH 7.8), 0.004% phenol red, and 0.5% sodium pyruvate according to Hannan (7) was used to determine MIC values. Tylosin (tylosin tartrate, Sigma) is water soluble and prepared by adding the required amount of distilled water. The tylosin was added to the columns of the plate according to the principle of doubling the dilution. Different concentrations ranging from 0.25 to 64 µg/ml were chosen depending on the efficacy of the antimicrobial agent (15). Briefly, the final volume (0.1 ml) of the twofold serially diluted antimicrobial was prepared in 96-well plates. Afterward, 0.1 ml of the mycoplasma inoculum was added to each well of the antibiotic dilutions. The number of viable germs of each mycoplasma test inoculum was examined simultaneously with the MIC test to verify the inoculum test in the range of 10^3 and 10^5 CCU/ml. The well with the uninoculated medium was considered a sterile control, the color-changing medium was an endpoint control, and the drug-free inoculum was a growth control. The microdilution plate was sealed with adhesive film with viability count for liquid MIC assays and incubated at 36±1°C until the color matched the endpoint control. For this purpose, the plates were checked frequently in the morning, noon, and afternoon. For the MIC value, the plates were incubated again to the endpoint in liquid test layers. Thus, a second reading was taken as the final MIC. Final values were determined 14 days later in slow-growing MG samples. To measure the initial MIC permanence and the minimum drug concentration at which no color change was observed, the plates were incubated until color changes occurred (7).

3. Results

It was found that 24 (63.1%) farms were positive in the serum plate agglutination test and 21 (55.2%) farms were positive in the ELISA test. Of the 38 broiler breeder flocks, 9 (23.68%) tested positive for Mycoplasma in culture, and fried-egg colonies appeared on the agar media and detected Mycoplasma species (Figure 1). The Mycoplasma colonies were detected under the microscope, and then an isolated colony was selected for inoculation into the broth.

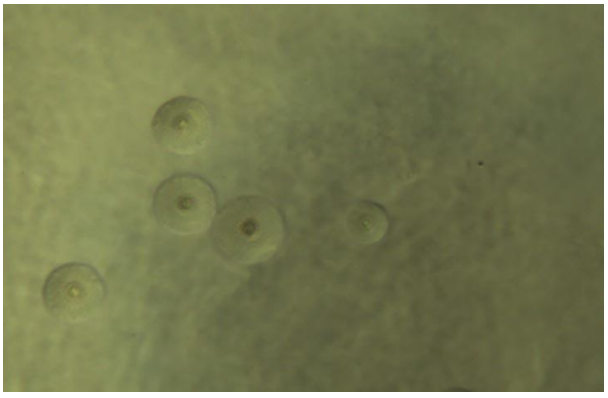

Figure1. Showing fried egg-shaped colonies on the surface of an agar plate

A 0.45-µm syringe filter (Nunk, Denmark) was used to filter the growth followed by replating on Frey's media agar. The process was performed in triplicate to obtain a pure culture of Mg isolates. In this study, 8 (21.05%) isolates were positive for MG, showing a specific amplicon at 531bp. PCR amplicons were prepared by electrophoresis in 1% agarose gel and a 100bp DNA ladder, and tested as negative controls (Figure 2). PCR identification of Mycoplasma species

in the total enriched samples was successful in 9 isolates, showing a specific amplicon at 163bp.

The MIC50 and MIC90 values were determined as the lowest concentrations that could inhibit the growth of isolates by 50% and 90%, respectively, according to the descriptions of Hannan (7). The results showed that tylosin caused MIC50 = 4 and MIC90 = 8. Table 2 tabulates MICs of tylosin against 8 isolates from MG.

The logarithm of the MIC values was summed for each strain and divided by the strains tested to calculate the geometric mean MIC, and then a mean MIC of 5.75 was obtained in this study. Compared with the MIC limits determined by Hannan (7), one isolate was sensitive to tylosin, three isolates were intermediate sensitive, and 50% of the isolates were resistant to tylosin. Our results from Iran were consistent with other geographic areas and suggested an increase in antibiotic resistance to tylosin (Table 3).

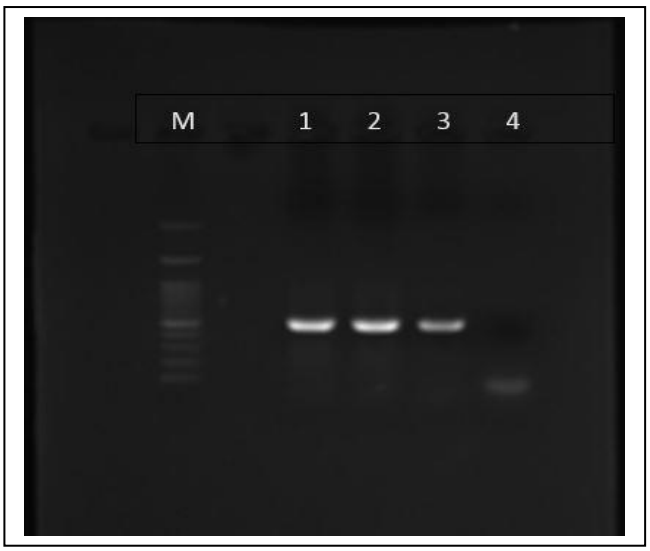

Figure 2. PCR electrophoresis analysis in % one gel agarose. M: Marker (100bp DNA ladder), *Lane 1:* Positive control (531bp band). *Lane 2* and *Lane 3* positive samples of some field isolates, *Lane 4:* Negative control (uncultured PPLO broth).

Table 2. Minimum inhibitory concentrations of tylosin (n=8)

Number of Mycoplasma gallisepticum isolates with MIC values (mg/ml)									Range	Mean MIC	MIC50a	MIC90b
0.25	0.5	1	2	4	8	16	32	64				
0	0	0	1	3	4	0	0	0	2–8	5.75	4	8

a Minimum inhibitory concentration of 50: antimicrobial concentration that inhibits at least 50% of the isolates
b Minimum inhibitory concentration 90: antimicrobial concentration that inhibits at least 90% of the isolates

Table 3. MIC values of tylosin and several field isolates in different studies with a high prevalence of resistance

Study	Range	MIC50a	MIC90
Present Study	2–8	4	8
Bradbury, Yavari (16) (UK)	0.125_ 16	0.125	4
Hannan (7) (USA, UK, JA, FR, DE, GE)	0.0025–10	0.01	2.5
Behbahan, Asasi (14) (Iran)	0.097_0.39	0.195	0.39
Gerchman, Levisohn (17) (IS)	0.0032-5	0.05	2.5
Gharaibeh and Al-Rashdan (18)	0.031–4	0.125	4
Elbehiry, Al-Dubaib (19) (SAUDI ARABIA)	--	0.032	1
Khatoon, Afzal (20) (PAKISTAN)	--	6.25	12.5
Gautier-Bouchardon (8)	0.03–5	--	--

4. Discussion

In this study, RSA, PCR, and culture methods were used to detect MG infection in broiler breeder farms. Samples were obtained from air sacs and pericardial regions by swabbing tracheas and homogenizing lung tissues of chickens in Mazandaran and Golestan provinces. The results confirmed MG infection of farms in the north of Iran. There is a need for efficient MG culture and molecular methods for diagnostic, investigative, and eradicative purposes. The activities of tylosin were tested *in vitro* against these isolates by the broth microdilution method, and it also showed that field isolates from MG altered their sensitivity to tylosin, demonstrating the need for a routine to optimize treatment doses and the efficiency of tylosin's MIC. As described in the literature, considering age-related variation in body clearance reduced interindividual variability in AUC (16).

Sarkar, Rahman (17) investigated the seroprevalence of MG in Bangladesh using the SPA test and reported 66.50% in laying hen farms and 58.9% in breeding poultry farms. Kapetanov, Orlic (18) showed that the overall seroprevalence of MS and MG in Serbian flocks was 47.49% and 9.01 in 2000 and 22.17% and 11.59 in 2009, respectively. For this purpose, they applied SPA and ELISA tests and found a decrease in the seroprevalence of MS and MG. Ventura Politte (19) measured the seroprevalence of MG in 40 commercial farms in the central and northern regions of Iran using the SPA method and reported that 4 out of 40 (10%) herds had infection with MG. These results were in line with our results and demonstrated the power of MG and the potential use of the SPA test as a rapid and cost-effective method. Serological ELISA and RSA tests are a good choice for monitoring and detecting MG infection in poultry flocks.

Molecular techniques, such as PCR, are a reliable choice given the disadvantages of culturing methods, such as the slow process, risk of contamination, and need for antibiotics. Yilmaz, Timurkaan (20) detected MG and MS in chickens using PCR, immunohistochemical, and cultural methods. For isolation of MG, 3 of 27 (11.1%) seropositive MG were used, and only 4 were found positive by the immunohistochemical method and PCR. Here, the agreement between culture and PCR was 88.8% in each case. Jafar and Noomi (21) also detected MS and MG using culture and PCR techniques. The rate of isolated mycoplasma was 35.1% and the sensitivity of culture was 100%, whereas the specificity of culture compared with PCR results was 97.9%. Feberwee, Mekkes (22) compared PCR, culture, and various serologic tests for the detection of MG and showed that the level of false positives changed with various serologic tests; therefore, the test is not recommended as the sole test and positive results should be confirmed by routine microbiologic testing or PCR.

Tayebi, Pourbakhsh (23) attempted to identify and isolate MG in the city of Ghaemshahr (northern Iran) and indicated that 20% of the samples were positive for the *Mycoplasma* genus and 12% of the farms were positive based on molecular testing. In addition, the findings of several researchers have shown that there is a high rate of MG in poultry farms in Iran regardless of the screening methods. According to Jalaladdini, Pourbakhsh (24), the PCR results of the infected samples from Kerman province (Iran) indicated the higher sensitivity of PCR compared to culture; therefore, PCR can be a more reliable approach for screening MG infections in poultry. These results were in agreement with those of the present study, and commercial farms in Iran were infected with MG.

In the past, chicken farmers used tylosin in water to feed broilers and brooders to inhibit the clinical signs of MG. However, MG has been reported to resist some antibiotics previously administered for mycoplasma infections in several cases. It has been shown that MG is effective *in vitro* against some antimicrobial agents, such as macrolides, tetracyclines, and fluoroquinolones. Based on the results, they are safe from other antimicrobial agents, such as penicillins and cephalosporins, which act by interfering with the

biosynthesis of cell dividers (6). Microbiologists have not still reached a common approach for testing mycoplasmas for antibiotic susceptibility. In the present work, MIC was determined by microbroth dilution because this method is generally simple and quick to perform, requiring only small amounts of media (7).

The activities of oxytetracycline, danofloxacin, and tylosin with the 10 strains of MG *in vitro* were studied by Bradbury, Yavari (25), and they showed that among 9 strains, oxytetracycline had the highest MIC values, and the tylosin had the lowest (MIC50=0.125 and MIC90=4, respectively). One strain showed resistance to tylosin with an MIC of 12. Nevertheless, the results showed that the isolates from MG had higher resistance to tylosin. Our results were inconsistent with those reported by Whithear, Bowtell (26). They used a microbroth dilution strategy for the MIC test in their study. Gerchman, Levisohn (27) determined the *in vivo* resistance to macrolides of 50 strains of MG, isolated in Israel between 1997 and 2010, and found that they detected tylosin and tilmicosin resistance in 50% of them. In addition, 72% (13.18) of strains included in clinical trials since 2006 had tylosin, enrofloxacin, and tilmicosin resistance. According to data provided by Gerchman, Levisohn (27), MG field strains with MICs of ≥ 0.63 μg/ml are considered resistant to tylosin strains. Although the results of our study were larger than those of Gerchman, Levisohn (27), they were consistent with respect to the increase in tylosin resistance.

In several studies, low MIC50 and MIC90 values of ≤ 0.125 μg/ml and ≤ 4 μg/ml for tylosin were found in isolates from MG, such as in research by Gharaibeh and Al-Rashdan (28) and Elbehiry, Al-Dubaib (29). In addition, Pakpinyo and Sasipreeyajan (30) determined very low values for the MIC range, and these results contradict those of in the present work. In Pakistan, Khatoon, Afzal (31) studied the susceptibility of some common antibiotics, such as tilmicosin, tylosin, erythromycin, and enrofloxacin to MG in commercial chicken flocks. In the mentioned study, tylosin showed MIC50 and MIC90 values of 6.25 μg/ml and 12.5 μg/ml, respectively, which were on average 56.25% higher than those reported in our study. In a review article, (8) compared the breakpoints reported by Hannan (7) and the Clinical and Laboratory Standards Institute (8) with the MIC of field isolates in the included studies and showed that resistant isolates were more common with both ceftiofur and amoxicillin (100%) and in descending order with erythromycin (54%), enrofloxacin (53%), oxytetracycline (47%), and tylosin (29%). Consistent with other reports and MIC values of reference strains (R=0.04 F=0.032 S6=0.022 PG31=0.024), the MIC of tylosin is one of the highest values observed in ineffective drugs in the field. It can be said that the cultivation of microorganisms in liquid media indicates the MIC, the lower value of which demonstrating that the drug is less necessary to inhibit the growth of the organism. Thus, the effective antimicrobials are the drugs with lower MIC values. The effective antimicrobials are the drugs with lower MIC values.

Few studies have been performed *in vitro* to evaluate the susceptibility of MG isolates to antimicrobial macrolides in Iran (32-34). Ghalehgolab Behbahan, Asasi (14) determined the *in vitro* activities of some antimicrobials, such as tiamulin and tylosin, against 28 isolates of MG, using the microbroth technique, the results of which ranged from 0.097 to 0.39, which was much higher in comparison to our result. There is a high risk of antibiotic resistance in farms in Iran. Since further information is needed, it is not easy to make a comparison between the antibiotic resistances of MG groups. In general, the current studies revealed higher MIC ranges and MIC90 and MIC50 values compared to those reported in the available literature. The current trend is indeed worrisome (8). Nonetheless, the valuable information may be useful in determining how far MG is likely to spread in farms. Thus, they can help researchers take better preventive measures against the spread of MG. For example, commercial farms must be routinely checked for infection with MG, and reactor birds must be culled because MG organisms can be transmitted vertically. Poultry specialists and

veterinarians can use the findings to provide therapeutic solutions for CRD and other mycoplasma diseases in poultry. There is still a need for the identification and isolation of mycoplasmas as well as the identification of antimicrobial susceptibility testing, which will help determine the best effective chemotherapy for CRD.

In summary, the ELISA and RSA tests have been shown to be less sensitive; however, they can be used as quickly and effectively as routine tests. Microbiological testing should also confirm positive results by other techniques, such as culture and/or PCR. Therefore, it is important to consider the drug resistance of mycoplasmas, which represents the need for various antibiotic options. Various MG field isolates showed tylosin resistance. This is cautioned because there are not enough field isolates and no tests to standardize antibiotic susceptibility levels. Common antibiotics, including tylosin, tiamulin, enrofloxacin, and lincomycin, should be the subject of routine standard reporting of antibiotic MICs because different veterinarians have their own prophylactic and therapeutic strategies for MG infections. These initiatives can be useful tools to reduce the cost of MG infection prevention and treatment programs.

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Authors' Contribution

Study concept and design: K. K
Acquisition of data: K. K
Analysis and interpretation of data: S. A. P., K. K and T. Z. S.
Drafting of the manuscript: K. K.
Critical revision of the manuscript for important intellectual content: S. A. P. and T. Z. S.
Statistical analysis: K. K., S. A. P. and T. Z. S.
Administrative, technical, and material support: K. K.

Ethics

All ethical guidelines were respected in sampling the submitted article according to the ethics committee rules of the Science and Research Branch, Islamic Azad University, Tehran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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