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Study of Minimum Inhibitory Concentration Against a Local Field Isolates of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* from Egyptian Broiler and Layer Chicken Flocks

Mustafa Bastamy[1], Ismail Raheel[2], Hany Ellakany[3] and Ahmed Orabi[4*]

[1]Department of Poultry and Rabbits Disease, Faculty of Veterinary Medicine, Cairo University, Egypt
[2]Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Beni-Suief University, Egypt
[3]Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Damanhour University, Egypt
[4]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Egypt
***Corresponding author:** drorabi2012@yahoo.com; orabivet@cu.edu.eg

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ABSTRACT

On a field level among poultry flocks, the efficacy of the most common antibiotics against *Mycoplasma* species was decreased, Hence the traceability of the new updates about the minimum inhibitory concentration becomes very important for veterinarians in fighting antibiotic resistant strains circulating among birds. In the current study the minimum inhibitory concentrations (MICs) of the common anti-mycoplasma drugs as enrofloxacin, difloxacin oxytetracycline, doxycycline, chlortetracycline, tylvalosin, erythromycin, tylosin, tilmicosin, spiramycin, tiamulin, lincomycin, spectinomycin and dihydrostreptomycin against MG and MS isolates of broiler and layer chickens in Egyptian farms and recorded in GenBank. The recovered results showed that till now the tylvalosin macrolides is the most efficient drugs in the control of mycoplasmas as it has the lowest MICs value against local *M. gallisepticum* and local *M. synoviae* as tylvalosin at dilution rate of 0.001-2µg/mL, showed the lowest values among the studied antibiotics as MICs value were 0.001,0.005 and 0.008µg/mL against MG and 0. 2,0.25 and 0.5µg/mL against MS strains.

Key words: Minimum inhibitory concentrations, *M. gallisepticum* (MG), *M. synoviae* (MS), Macrolides, Tylvalosin, Broiler chickens, Layers chickens.

INTRODUCTION

Avian *Mycoplasma* infection is extremely important to both the broilers and layers as it is worldwide disease possibly due to the growth of large flocks with in little geographically areas, increase stock density, under inadequately biosafety conditions at which *Mycoplasma* can affect grower poultry, generating important economic losses (OIE 2007; OIE 2008; Dufour et al. 2006). The most important pathogens associated with avian mycoplasmosis were *M. gallisepticum* and *M. synoviae*. *M. gallisepticum* producing, an infectious contagious avian respiratory disease with a large range of clinical lesions as increase of mortality%, decrease eggs and meat production, decrease of fertility and hatchability %, combined with high cost of treatment and control (Lockaby et al. 1998). *M. synoviae* is considered the main agent associated with avian infectious synovitis, which occurs in chickens and turkeys (Ramirez et al. 2006; Bosila et al. 2021). The prophylactic measures against avian mycoplasmosis are carried to obtain *Mycoplasma* free flocks. The prophylaxis is based on technological, hygienical and nutritional factors as; avoidance of stressors; discarding the other infectious diseases which may favors the evolution of mycoplasmosis; regular disinfections in hatcheries and farms; treatment of fertile eggs with antibiotics; treatment with antibiotics in the first days of life chicken against *Mycoplasma* as a preventive measure (Valks and Burch 2002). Economic losses caused by Mycoplasma's infection in chicken and turkey flocks, solely or in conjunction with other organisms are high and associated with an increase of condemnation rate, less final weight and decrease of egg production between 5 and 10%, poor feed conversion ratio (Kapetanov et al. 2010; Bradbury 2001). Although antibiotics are commonly used to reduce the effects of Mycoplasmas infections, they are ineffective at clearing *M. gallisepticum* lesions (Ley and Yoder 1997; Elazab et al. 2021). Mycoplasmas are resistant to β-lactams antibiotics

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because of the absence of cell-wall structure, so the drug of choice for the therapy for mycoplasmosis were fluroquinolones, tetracyclines, macrolides and pleuromutilin which induce DNA fragmentation or inhibition of protein synthesis (Behbahan et al. 2008). Macrolide antibiotics are the most common anti-mycoplasma agents used in Egyptian flocks that are classified as macrocyclic lactones with 12-20 carbon atoms in the lactone ring at which several combinations of de-oxy sugars can be attached by glycosidic linkages (Watteyn et al. 2013). Acetyl-iso-valeryl-tyrosine tartrate (Tylvalosin /Tylvamyco®) is a member of the macrolide group commonly used in poultry farms for the treatment of respiratory infections specially the treatment of mycoplasmosis at the recommended dose 25mg.kg^{-1} for 3 days (Forrester et al. 2011; Bastamy et al. 2020). Mycoplasmas have mutation rates higher than conventional bacteria which means that they can rapidly develop resistance to other drugs including the tylosin and oxytetracyclines as has been reported in Europe (Ahling et al. 2000). The massive use of anti-mycoplasma agents led to the emergence of resistant *Mycoplasma gallisepticum* and *Mycoplasma synoviae* strains (Gautier-Bouchardon et al. 2002). However, the carrier state of infected poultry is not eliminated by drugs application but only suppresses the excretion in respiratory secretions and eggs (Stipkovits 2000). Few reports on the minimum inhibitory concentration values for avian mycoplasmas are available in literature, despite in the last years, the need of updated data become urgent (Lysnyansky et al. 2013), As knowing the antibiotic susceptibility of the circulating strains is very important for better managing the drug therapy, so the present investigation aimed to focus on the current antimicrobial susceptibility profiles of local *Mycoplasma* gallisepticum and *Mycoplasma synoviae* recovered from broiler and layer flocks in Egypt.

MATERIALS AND METHODS

Mycoplasma Isolates

The examined isolates used in this study were *M. gallisepticum* (MG) recovered from broiler chickens suffered from respiratory complains. MG isolate accessed on GenBank and coded as; BankIt2433660 MG-EGY/ORABI/Raheel/2020 MW679029, *M. synoviae* (MS) isolate recovered from arthritis of layer chickens and accessed on GenBank coded as; BankIt2433662 MS-EGY/ORABI/Raheel/2020 MW679030.

In Vitro Cultivation of *Mycoplasma* Isolates

The examined *M. gallisepticum* and *M. synoviae* isolates were re-cultured and subcultured on Frey's broth, and adjusted finally to 10^5cfu/mL followed by inoculation onto an agar plate of Avian Mycoplasma Agar for regular microscopic examination (Behbahan et al. 2008).

In vitro Preparation of Antimicrobials

Antimicrobial agents used during this study originated from Sigma-Aldrich, Germany as the following: fluoroquinolones: Difloxacin (DIF) and Enrofloxacin (ENR); aminoglycoside: Spectinomycin (SPC) and Dihydro-streptomycin (DHS); lincosamides: Lincomycin (LCM); the tetracyclines: doxycycline (DOX), chlortetracycline (CTC) and oxytetracycline (OTC); the Macrolides: Tylvalosin (TVN) (Tylvamyco®) was obtained as 62.5% water-soluble white granules (ATCO distributed by MN trade company, Egypt) at which Each gram powder contains 625 mg of TVN as TVN tartrate., Tilmicosin (TIL), Tylosin (TYL), Spiramycin (SPM) and Erythromycin (ERY); and pleuromutilin: Tiamulin (TIA).The antibiotics were prepared according to the recommendations of producer and dilutions of the antibiotics were freshly prepared for each agent from the aliquots stored at −70°C and two fold serial dilutions were prepared for detection of MICs values (Hannan 2000).

Determination of Minimum Inhibitory Concentration of MG and MS

According to (Hannan 2000; CLSI 2011), the clinical isolates were tested on each 96 well microtiter plates by using the micro broth dilution methods at which 10^4–10^5cfu/mL of the strains were performed in mycoplasma broth medium with controlled pH value (broth medium adjusted to pH 6.8). MIC values were determined from the lowest concentrations of the antibiotics where no pH and color change of the broth was detected after one week of incubation, while MIC_{50} and MIC_{90} values were defined as the lowest conc. that inhibited the growth of 50% or 90% of the strains, respectively.

Statistical Analysis

GLM procedure used for Data analysis and the means were compared following the Tukey's method of SAS (SAS Institute 2008). which depend mainly on calculation of the averages and the standard deviation between the recorded data.

RESULTS

The results in Table 1 and Fig. 1 and 2 showed that the MICs (MIC, MIC_{50} and MIC_{90}) (µg/mlL values against examined *M. gallisepticum* (MG) MW679029 and *M. synoviae* (MS) MW679030 were as the following: fluoroquinolones as enrofloxacin at a dilution range 0.1-2µg/mL, the MIC, MIC_{50}, and MIC_{90} were 0.2, 0.6 and 1.32µg/mL against *M. gallisepticum* and 0.4,0.7and 1.5µg/mL against *M. synoviae*, while difloxacin at the same dilution range the MICs value were 0.2, 0.7 and 1.3µg/mL against MG and 0.4, 0.7 and 1.6µg/mL against MS. Aminoglycosides as spectinomycin at dilution range 0.5-16µg/mL showed MICs values 3.6, 5.4 and 8.6 against MG and more than 16µg/mL against MS, while dihydrostreptomycin values were more than 16µg/mL against MG and MS, in the other hand lincosamides as lincomycin at the same dilution range slowed MICs values of 4.6,12.9 and more than 16µg/mL against MG ,while *M. synoviae* MICs values were more than 16µg/mL. Tetracyclines as oxytetracyclines and chlortetracycline at dilution range 0.5-8µg/mL showed MICs values ranged from 0.5, 0.7 and 1.9µg/mL against MG and 0.5, 0.7 and 2µg/mL against MS, while doxycycline at dilution range 0.1-2µg/mL showed 0.3,0.6 and 1.4 against MG and 0.2, 0.6 and 1.6µg/mL against MS. Macrolides as tylvalosin at dilution range 0.01-2µg/mL showed the lowest values among the studied antibiotics as MICs value were 0.001, 0.005 and 0.008µg/mL against MG and 0.02, 0.25 and

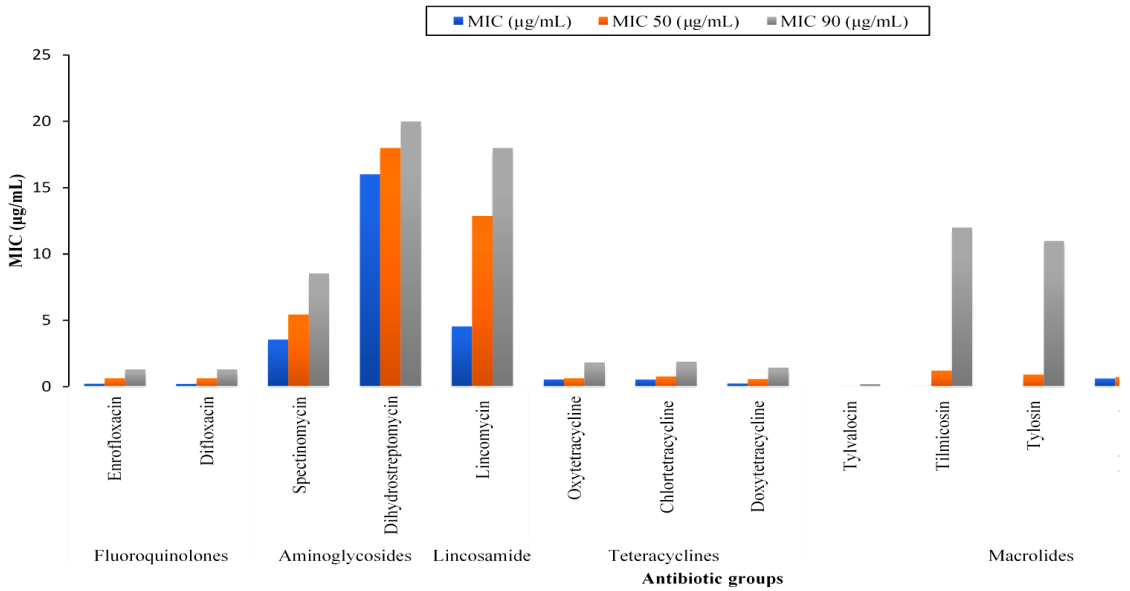

Fig 1: MICs value of the common anti-mycoplasma drugs against local Egyptian *M. gallisepticum*

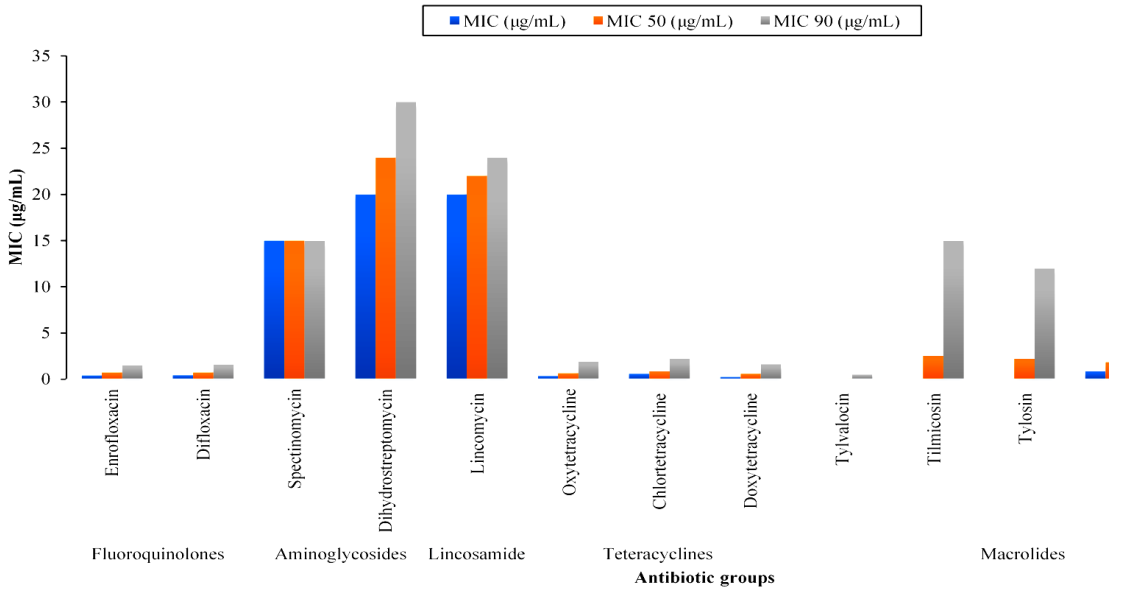

Fig. 2: MICs value of the common anti-mycoplasma drugs against local Egyptian *M. synoviae*

Table 1: MICs values of the common anti-mycoplasma drugs against local Egyptian *M. gallisepticum* and *M. synoviae*

Antibiotic Groups	Antibiotic members	Dilution range (µg/mL)	Minimum inhibitory concentration values (µg/mL)					
			M. gallisepticum			*M. synoviae*		
			MIC	MIC $_{50}$	MIC $_{90}$	MIC	MIC $_{50}$	MIC $_{90}$
Fluoroquinolones	Enrofloxacin	0.1–2	0.23	0.64	1.32	0.42	0.72	1.52
	Difloxacin	0.1–2	0.22	0.65	1.32	0.44	0.73	1.58
Aminoglycosides	Spectinomycin	0.5–16	3.55	5.44	8.55	>16	>16	>16
	Dihydrostreptomycin	0.5–16	16	>16	>16	>16	>16	>16
Lincosamide	Lincomycin	0.5–16	4.55	12.88	>16	>16	>16	>16
Teteracyclines	Oxytetracycline	0.5–8	0.56	0.65	1.84	0.35	0.65	1.88
	Chlortetracycline	0.5–8	0.55	0.78	1.88	0.58	0.85	2.21
	Doxycycline	0.1–2	0.25	0.58	1.44	0.23	0.58	1.62
Macrolides	Tylvalosin	0.001–2	0.001	0.005	0.008	0.02	0.25	0.5
	Tilmicosin	0.01–16	0.06	1.22	12	0.08	2.52	15
	Tylosin	0.01–16	0. 06	0.92	11	0.08	2.2	12
	Spiramycin	0.5–8	0.62	0.75	4.24	0.84	1.85	4.52
	Erythromycin	0.5–8	8	>8	>8	>8	>8	>8
Pleuromutilins	Tiamulin	0.01–2	0.07	0.55	1.42	0.08	0.65	1.78

0.5µg/mL against MS, while tilmicosin and tyrosine at dilution range 0.1-16µg/mL their values were 0.06, 1.2, 12 and 0.06, 0.9, 11µg/mL respectively against MG and 0.08,2.5,15 and0.08,2.2,12µg/mL against MS. In the other hand spiramycin at dilution range 0.5-8µg/mL showed MICs value 0.6, 0.8 and 4.4µg/mL against MG and 0.8, 1.9 and 4.5µg/mL against MS, while erythromycin at dilution range 0.5-8µg/mL showed MICs value more than 8µg/mL against MG and MS. Pleuromutilin as timulin at dilution range 0.01-2µg/mL showed MICs value 0.07, 0.6 and 1.4µg/mL against MG and 0.08, 0.7 and 1.8 against MS.

DISCUSSION

Mycoplasma are found on mucosal surfaces of the respiratory tract of the birds so it needs a host to be live and persist for short time in the external environment (Kapetanov et al. 2010). *M. gallisepticum* is the causative agent for chronic respiratory disease in broiler chickens, the disease not only causes losses in weight gain, disturbance in feed conversion efficiency, elevation in mortality rate and severe condemnations in the slaughterhouses, but also transmitted horizontally and vertically and the flock remain suffering from subclinical infections (Gharaibeh and Al Roussan 2008; Markey et al. 2013). There are many predisposing factors that affect mycoplasmas occurrence among broiler and layer chicken flocks as the immune condition of birds, litter quality, overcrowdings, adverse climate, fomites and workers have a role in transmission and infections (Nneoma Okwara 2016). Low pathogenic avian influenza mixed infection with MG in chickens has been recorded (Stipkovits et al. 2012; Sid et al. 2016), also infection with IBV, *E. coli* or other pathogens make disease more serious (Matilda et al. 2018; Nneoma Okwara 2016). Mycoplasmas pathogenesis shows that it is a facultative intracellular organisms, which can adhere to host target cells, stimulate apoptosis (Nascimento 2000). *M. gallisepticum* prevalence poultry flocks in developing countries poultry flocks as Malaysia was 43% layer and 64% broiler (Ching et al. 2016), 45.1, 53.4 and 29.5% in Bangladesh and Pakistan and Ghana, respectively (Hossain et al. 2010; Hussain et al. 2018; Matilda et al. 2018; Peebles and Branton 2012), while in Belgium the incidence of MG Layers flocks was 0.9 and 2.7% in broilers chickens (Michiels et al. 2016). Mostly subclinical *M. synoviae* infection in wild birds which characterized by respiratory and arthritis signs resulting in pneumonia, synovitis and bursitis mainly with egg deformity as thinning, breaks and cracks (Ferguson and Noormohammadi 2013; Feberwee et al. 2009). In the current study the local isolate *M. gallisepticum* recovered from broiler chickens suffered from respiratory signs and accessed on GenBank coded MW679029, while *M. synoviae* isolate was recovered from arthritis in layer chickens and accessed on GenBank coded MW679030. The two isolates were examined against most common anti-mycoplasmas drugs as fluoroquinolones: Difloxacin and Enrofloxacin; aminoglycoside: Spectinomycin and Dihydro-streptomycin; lincosamides: Lincomycin; the tetracyclines: doxycycline, chlortetracycline and oxytetracycline; the Macrolides: Tylvalosin, Tilmicosin, Tylosin, Spiramycin and Erythromycin; pleuromutilins: Tiamulin. Treatment of mycoplasma-infected flocks with antibiotics decreases the clinical signs and the risk of transovarian transmission (Ortiz et al. 1995; Stipkovits and Kempf 1996). Mycoplasmas are resistant to antibiotics that act on cell wall peptidoglycan, such as penicillin, but are sensitive to tetracyclines, macrolides and quinolones as these drugs accumulate in high concentrations in the mucosal membranes of the respiratory and genital tracts (Nascimento et al. 1999; Stipkovits and Kempf 1996; Hannan et al. 1997). The present study aimed at updating the MICs data of the local MG and MS isolates recovered from Egyptian flocks and the results in Table 1 and Fig . 1 and2 showed that enrofloxacin and difloxacin at a the same dilution range, mainly have nearly MICs values against MG and MS as the following; MIC, MIC_{50}, and MIC_{90} were 0.2, 0.6 and 1.32µg/mL against *M. gallisepticum* and 0.4,0.7 and 1.5µg/mL against *M. synoviae*, while only spectinomycin from aminoglycosides group showed valuable results dilution range 0.5-16µg/mL which were MICs values 3.6,5.4 and 8.6 against MG and more than 16µg/mL against MS, in the other hand lincomycin at the same dilution rate slowed MICs values 4.6, 12.9 and more than 16µg/mL against MG, while *M. synoviae* MICs values were more than 16µg/mL. Doxycycline act as the potent agent against Mycoplasmas among tetracyclines group as at dilution range 0.1-2µg/mL showed 0.3, 0.6 and1.4 against MG and 0.2, 0.6 and 1.6µg/mL against MS, while oxytetracyclines and chlortetracycline at dilution range 0.5-8µg/mL showed MICs values ranged from 0.5, 0.7 and 1.9µg/mL against MG and 0.5, 0.7 and 2µg/mL against MS. The truth is not denied that the antibiotic susceptibility assay is predicted in vivo success or failure of therapy through measuring the growth response of an isolated organism to specific agents (Bradbury and Morrow 2008). In the present study the Macrolides as tylvalosin at dilution range 0.001-2µg/mL showed the lowest values among the studied antibiotics as MICs value were 0.001,0.005 and 0.008µg/mL against MG and 0.02, 0.25 and 0.5µg/mL against MS, while tilmicosin and tylosin at the same dilution range, their values were 0.06, 1.2, 12 and 0.06, 0.9, 11µg/mL respectively against MG and 0.08, 2.5, 15 and 0.08, 2.2, 12µg/mL against MS. In the other hand spiramycin at dilution range 0.5-16µg/mL showed MICs value 0.6, 0.8 and 4.4µg/mL against MG and 0.8, 1.9 and 4.5µg/mL against MS, while erythromycin at dilution range 0.5-8µg/mL showed MICs value more than 8µg/mL against MG and MS. Finally, pleuromutilin as timulin at dilution range 0.01-8µg/mL showed MICs value 0.07, 0.6 and 1.4µg/mL against MG and 0.08, 0.7 and 1.8 against MS. Unfortunately, the minimum inhibitory concentrations of antimicrobials in vitro studies shown resistance to some macrolides and enrofloxacin, while no resistance to tiamulin or tylvalosin could be evidenced in *M. gallisepticum* or *M. synoviae*, but mutants *Mycoplasma* spp. that became resistant to tylosin were also resistant to erythromycin, whereas mutants resistant to erythromycin were not always resistant to tylosin (Gautier-Bouchardon et al. 2002). A study in Israel collected during 2005–2006 indicated decrease in susceptibility against enrofloxacin and difloxacin compared with archived strains (1997–2003) (Gerchman et al. 2008), this is agreeing with a study from Jordan at which compared MICs of *Mycoplasma* isolates recovered from 2004 to 2005 vs. strains isolated during 2007–2008 confirmed a significant increase in MIC

values (Gharaibeh and Al-Rashdan 2011). A new macrolides agent as tylvalosin is the useful economic drug in the treatment and control of *Mycoplasma* spp. infection and tiamulin from pleuromutilin group. However, tiamulin medication is contraindicated in flocks with ionophore antimicrobials, since it may lead to toxicity (Horrox 1980). In conclusion studying MICs values variation will be fundamental in order to create a significant database that would direct veterinarians in selecting the proper drug for treating these impactful *Mycoplasma* infections. The current study proved that till now the tylvalosin macrolides is the most efficient drugs in controlling MG and MS as it has the lowest MICs values.

Author's Contribution

All authors contributed equally to study the design methodology, interpretation of results, and writing of the manuscript.

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