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[*Comunicação*]

The first assessment of antimicrobial sensitivity of *Mycoplasma gallisepticum* and *M. synoviae* from laying hens in Brazil

[*Primeira avaliação do perfil de susceptibilidade antimicrobiana de Mycoplasma gallisepticum e M. synoviae de poedeiras comerciais no Brasil*]

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Avian mycoplasmosis have significant economic implications in poultry production, hence it holds prominence among avian diseases of health importance for the sector. *Mycoplasma gallisepticum* and *M. synoviae* stand as the primary pathogenic species within the genus, capable of inducing clinical or subclinical illnesses in birds. These two species feature on the World Organization for Animal Health (Terrestrial…, 2023) list of notifiable diseases and are among the maladies prioritized in the National Poultry Health Program (NPHP) of the Ministry of Agriculture and Livestock (Brasil, 2001). Lesions triggered by mycoplasmas are primarily confined to the respiratory tract, although they may also occur in the joints and, in some instances, in the urogenital tract, leading to losses such as decreased egg production and quality due eggshell apex abnormalities, increased mortality, including embryonic death in fertile eggs, high rates of chick culling, and condemnation of carcasses for slaughter (Nascimento *et al*., 2020).

For the diagnosis of infections caused by mycoplasmas, it is important to combine analyses of epidemiological data, observation of clinical signs, evaluation of macroscopic and microscopic lesions, as well as detection of the agent through isolation and/or Polymerase Chain Reaction (PCR) (Nascimento *et al.*, 1991; Nascimento *et al.*, 2020). Isolation and identification of the agent are considered the "gold standard" for mycoplasma infection diagnosis and require specific culture media, with the Frey medium being widely used, where they grow in colonies resembling either mammillary or fried egg-like structures when cultured on solid media (Beylefeld *et al.*, 2018; Nascimento *et al.*, 2020).

Control of *M. gallisepticum* and *M. synoviae* infection is essential in all phases of poultry production due to the losses it can cause. The poultry industry employs a variety of strategies for controlling mycoplasmosis including antimicrobial macrolide administration (Morrow, 2024). However, the treatment and control of infections with antibiotics as a measure that can lead to drug resistance over time. Nowadays, antimicrobial resistance is a one heath challenge and poses a risk to compromise bacteria treatment in human and animal health (Pokharel *et al*., 2020).

Considering the importance of the correct use of antibiotics in animal health and the quality and safety of animal-derived products, the importance of expanding our understanding of mycoplasmosis, as well as their resistance to drugs used in the poultry sector, becomes increasingly evident. This study aims to investigate antimicrobial susceptibility in strains of *M. gallisepticum* and *M. synoviae.*

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A total of 105 tracheal samples from laying hens from flocks previously positive to *M. gallisepticum* and *M. synoviae* by PCR were analyzed. Samples were obtained in poultry farms located in the Midwest region of São Paulo State. The samples were placed in microtubes and stored in a freezer at -80°C at the Avian Health Laboratory of the Federal Fluminense University. The samples were cultured according to Nascimento *et al*. (2020) using both liquid and solid modified Frey medium. Specimens in liquid medium, displaying a yellowish color due to medium acidification, were subcultured onto solid medium for mycoplasma isolation. Colonies compatible with mycoplasmas (mammillary or fried egg-like) were cloned through three passages on solid and liquid media to obtain pure colonies. In each passage, aliquots were subjected to PCR for confirmation of genus and species, as previously described. For confirmation of the species PCR was employed as described by Nascimento *et al.* (2005) for *M. gallisepticum* and Lauerman *et al.* (1993) for *M. synoviae*.

For determination of the Minimum Inhibitory Concentration (MIC), the sample to be tested needed to be titrated and have a concentration of 10^5 Color Changing Units (CCU)/ml. CCU determination involved serial dilution (10^{-1} to 10^{-13}) transferring 0.1mL of the sample to 0.9mL of liquid Frey medium. Subsequently, 100µL of liquid Frey medium was transferred to each well of row A, wells 1 to 12, and 100µL of each dilution (10^{-2} to 10^{-13}) was added. For medium control, 200µL of liquid Frey medium was added to well 12 of row B. The plate was then sealed, incubated, and examined every 24 hours until the medium changed color, indicating bacterial growth. The CCU was determined by the lowest dilution of the culture showing a color change (Hannan, 2000).

MIC was conducted using the broth dilution method for the antimicrobials tylosin and tiamulin, employing 96-well microdilution plates (Methods…, 2011; Hannan, 2000). A stock solution of 128mg/ml of each antimicrobial agent was initially diluted in ethanol. The expected final concentration range of antibiotics was 0.0004 to 64mg/mL. For determination of MIC for each isolate, 25µL of modified liquid Frey medium was added to wells 2 to 12 of row A. Then, 25µL of each antimicrobial solution, at the highest concentration to be tested, was distributed into wells 1 and 2 of row A, followed by serial dilution at a ratio of 1:1, transferring 25µL from well 2 to well 3, and so forth until well 12, when the final 25µL was discarded. Finally, 175µL of the appropriately titrated sample was added to wells 1 to 12. All samples were tested in triplicate. For each strain tested, a positive control containing 25µL of sterile medium and 175µL of the isolate without antimicrobial was used. A negative control consisted of a well with 200µL of sterile medium without inoculum. As an antimicrobial control, a well with 175µL of sterile medium and 25µL of the antimicrobial was used. The plates were sealed, incubated, and examined every 24 hours over a period of 5 to 7 days until the last well where color change occurred matched that of the positive control. MIC was defined as the lowest concentration of antimicrobial that prevented bacterial growth, when the control wells change color (Methods…, 2011).

Through culture, positive samples for *Mycoplasma* spp. were obtained in all batches, but there was difficulty in obtaining pure cultures of MG and MS due to the rapid growth of other species of mycoplasmas, considered commensal. This fact has been previously reported by Beylefeld *et al.* (2018), who found that non-pathogenic species were isolated more frequently than pathogenic species of avian mycoplasmas. Four strains of MG and one of MS were isolated and used for antimicrobial susceptibility testing.

In the evaluation of susceptibility profile to tylosin and tilmicosin, the MIC values for MG ranged from 0.0004mg/L to 0.003mg/L for tylosin and 0.0004mg/L to 0.007mg/L for tilmicosin. The MS strain had MIC values of 0.001mg/l for tylosin and 0.007 mg/L for tilmicosin (Table 1). Currently, there are no international standards for in vitro susceptibility testing for veterinary antimicrobial agents for mycoplasmas, and therefore the macrolide breakpoints provided by Hannan (2000) were used as parameters. Thus, the samples evaluated in this study were considered susceptible since the antibiotic concentration needed to inhibit growth was very low. As obtained in the present study low MIC values were reported for the tested antimicrobials in strains of *M. gallisepticum* and *M. synoviae* (Taiyari *et al.*, 2021).

Table 1. Antimicrobial susceptibility* of *Mycoplasma gallisepticum* and *M. synoviae* strains from commercial laying hens

Sample	Species	Minimal Inhibitory Concentration	
		Tylosin	Tilmicosin
1	*M. gallisepticum*	0,003 mg/l	0,007 mg/l
2	*M. gallisepticum*	0,0004 mg/l	0,001mg/l
3	*M. gallisepticum*	< 0,0004 mg/l	0,003 mg/l
4	*M. gallisepticum*	0,0004 mg/l	0,0004mg/l
5	*M. synoviae*	0,001 mg/l	0,007 mg/l

*Macrolide breakpoint: < 1mg/l (Hannan *et al*., 2000)

The appropriate selection and use of antimicrobial agents to effectively combat avian mycoplasmosis are fundamental for maintaining bird health. This requires knowledge of the antimicrobial resistance profile of avian mycoplasmas. The absence of defined standards for interpreting susceptibility test results underscores the urgent need to conduct studies aimed at defining specific breakpoints for antimicrobial dosing to be tested against avian mycoplasmas. Establishing specific interpretive criteria will provide veterinarians with valuable tools to optimize antimicrobial use in birds, thereby reducing selective pressure on these therapeutic agents and preventing the selection and spread of more resistant strains. Additionally, it will enable the formulation of more targeted and effective treatment options, ensuring the continuous health of birds and preserving the effectiveness of available antimicrobials.

Keywords: minimum inhibitory concentration, tylosin, tilmicosin

RESUMO

O objetivo deste estudo foi investigar o perfil de susceptibilidade antimicrobiana de Mycoplasma gallisepticum *e* M. synoviae *obtidos de galinhas poedeiras comerciais em relação à tilosina e tilmicocina. Cento e cinco amostras de traqueia de galinhas poedeiras previamente positivas para* M. gallisepticum *e* M. synoviae *por PCR foram analisadas. As amostras foram cultivadas em meio Frey modificado. Placas que exibiram colônias compatíveis com* Mycoplasma *spp. foram submetidas a clonagem por meio de três passagens sucessivas no mesmo meio para obter colônias puras. As cepas clonadas foram submetidas à PCR para confirmação de espécie e ao teste da Concentração Inibitória Mínima (CIM) à tilosina e tilmicosina. No cultivo, foram isoladas quatro cepas de MG e apenas uma de MS. Os valores da CIM de* M. gallispeticum *variaram de 0,0004 mg/L a 0,003 mg/L para tilosina e 0,0004 mg/L a 0,007 mg/L para tilmicosina.* M. synoviae *teve a CIM de 0,001 mg/L para tilosina e 0,007 mg/L para tilmicosina. A concentração de antibiótico necessária para inibir o crescimento de todas as amostras testadas foi muito baixa, sugerindo que o seu uso seria eficiente para o controle de infecção provocada por essas cepas de micoplasmas.*

Palavras-chave: concentração inibitória mínima, tilmicosina, tilosina

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