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Molecular detection and characterization of *Mycoplasma gallisepticum* and *M. synoviae* from Brazilian laying hens

Detecção molecular e caracterização de *Mycoplasma gallisepticum* e *M. synoviae* em galinhas poedeiras brasileiras

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Highlights

A high frequency of *M. synoviae* in laying hen flocks.
Molecular characterization of *M. gallisepticum* strains from Brazil.
The *M. gallisepticum* strains clustered into one group.

Abstract

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) are important pathogens in the poultry industry, causing substantial economic losses. Genotyping these pathogens is essential, as they exhibit variations in pathogenicity and impact on poultry flocks. This study aimed to detect the presence of MG and MS and to genotype isolates from Brazilian laying hens. A total of 172 tracheal swabs were collected from laying hens at different production stages. *Mycoplasma* detection was performed using PCR and culture methods. The *mgc2* gene was sequenced to genotype MG clones. Among the 172 samples, 43 (25.00%) tested positive for MG by PCR, while 123 (71.51%) tested positive for MS. Culture analysis identified four MG-positive flocks, whereas none tested positive for MS. PCR analysis revealed a high occurrence of both MG and MS. The strains demonstrated distinct clustering patterns, with our isolates forming closely related groups.

Key words: Mycoplasmosis. Laying hens. *mgc2*. Genotype.

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Resumo

Mycoplasma gallisepticum (MG) e *M. synoviae* (MS) são patógenos importantes na indústria avícola, causando perdas econômicas significativas. A genotipagem desses patógenos é crucial, uma vez que eles apresentam variações na patogenicidade e no impacto sobre os lotes de aves. Este estudo teve como objetivo detectar a presença de MG e MS e genotipar os isolados provenientes de galinhas poedeiras brasileiras. Um total de 172 suabes traqueais foram coletados de galinhas poedeiras em diferentes estágios de produção. A detecção de *Mycoplasma* foi realizada utilizando métodos de PCR e cultivo. O gene *mgc2* foi sequenciado para genotipar os clones de MG. Dentre as 172 amostras, 43 (25,00%) testaram positivo para MG por PCR, enquanto 123 (71,51%) testaram positivo para MS. A análise por cultivo identificou quatro lotes positivos para MG, enquanto nenhum teste foi positivo para MS. A análise por PCR revelou uma alta prevalência de MG e MS. Nossas cepas demonstraram padrões distintos de agrupamento, com nossos isolados formando grupos intimamente relacionados.
Palavras-chave: Micoplasmose. Poederias. *mgc2.* Genótipos.

Introduction

Mycoplasma gallisepticum (MG) and *M. synoviae* (MS) belong to the class *Mollicutes,* characterized by their small size and lack of a cell wall. Both pathogens cause respiratory diseases in poultry and are considered major threats to the poultry industry. Economic losses due to avian mycoplasmosis stem from decreased egg production and quality, poor hatchability due to high embryonic mortality, increased culling rates, reduced feed efficiency, high mortality rates, and elevated antibiotic treatment costs (Nascimento et al., 2020).

Although culture remains the gold standard for identifying these microorganisms, isolating MG and MS is challenging due to their fastidious growth requirements and susceptibility to overgrowth by non-pathogenic mycoplasmas (Fialho et al., 2023). PCR-based methods and immunoassays offer solutions to these challenges by providing more accurate results (Magalhães et al., 2020; Silva et al., 2021).

Genotyping MG is essential for understanding its genetic diversity and virulence potential. The *mgc2* gene encodes cytadhesin proteins, which are crucial for bacterial attachment to host cells. Due to its combination of conserved and highly variable regions, *mgc2* is widely used for MG genotyping. Studies have demonstrated its utility in characterizing MG strains and assessing their pathogenic potential (Ferguson et al., 2005; Limsatanun et al., 2022; Matucci et al., 2020).

Disease control relies on biosecurity measures, sanitation, antibiotic use, and vaccination. While MG vaccination is widespread in Brazil, with live vaccines such as MG-70 and MG-F being commonly used, MS vaccination is not a common practice (Silva et al., 2021a; Nascimento et al., 2005b).

This study aimed to investigate the presence of *M. gallisepticum* and *M. synoviae* and to genotype the clones isolated from laying hens in Brazil.

Material and Methods

Sampling

The study was approved by the Ethics in Animal Use Committee of Universidade Federal Fluminense (case no. 3831200919). A convenience sampling method was used in this study. Using swabs, 172 tracheal samples were collected from laying hens in the growing and production phases. The chickens belonged to 15 flocks from six different farms (A to F). On each farm, 11 to 13 chickens were sampled per flock. Tracheal swabs were aseptically collected and immediately transferred to sterile microtubes containing modified Frey's liquid medium supplemented with 50% glycerin. The samples were carefully stored at refrigeration temperature to maintain viability during transportation. Subsequently, the microtubes were transported to the Poultry Health and Molecular Epidemiology Laboratories at the Faculty of Veterinary Medicine, UFF, where they underwent further processing and analysis as described above.

Mycoplasma isolation

An aliquot of 0.2 mL of each collected sample was inoculated into 1.8 mL of modified Frey's liquid medium (Himedia, India). Serial dilutions were performed up to 10^{-5}, and the 10^{-3} and 10^{-5} dilutions were spread onto plates containing modified Frey's solid medium (Himedia, India) (Nascimento et al., 2020). All samples were incubated at 37 °C under microaerophilic conditions and observed for 21 days using a 100× magnification stereoscopic microscope (Razin & Tully, 1995). Egg-shaped colonies obtained from PCR-positive samples for MG and MS were selected and inoculated onto liquid and solid Frey medium. This procedure was repeated three times to ensure sample purity.

Molecular detection and identification

Both samples and isolates were subjected to PCR for the detection of MG and MS. A 0.5 mL sample aliquot was used for DNA extraction following the phenol-chloroform adapted method (Sambrook & Russell, 2006). The DNA quantity and quality were assessed using a BiodropTouch® spectrophotometer (Biochrom, Harvard Bioscience, USA). PCR was then conducted to detect MG and MS, following the protocols described by Nascimento et al. (2005a) and Lauerman et al. (1993), respectively. For MG detection, the first reaction mixture contained 1× PCR buffer, 0.2 mM dNTPs, 1 U Taq polymerase (Promega, Brazil), 0.2 µM of each specific primer, and 5 µL of DNA. The second reaction was conducted under the same conditions, except that 5 µL of the amplicon from the first step was used in place of DNA. For MS detection, the reaction mixture contained 1× PCR buffer, 0.2 mM dNTPs, 0.2 µM of each specific primer, 1 U Taq polymerase (Promega, Brazil), and 5 µL of DNA. All reactions were performed in a PTC-100® thermocycler (Bio-Rad Laboratories, England) with a final volume of 25 µL (Table 1). The MS ATCC 25204 and MG ATCC 129 S6 strains were used as positive controls, while ultrapure water was used as the negative control. The PCR amplicons were separated on a 1.5% agarose gel submerged in Tris-Borate-EDTA (TBE) buffer and subjected to electrophoresis at 94 V for

40 min. After electrophoresis, the gel was stained with 0.5 µg/mL of ethidium bromide (Ludwig Biotec, Brazil), and the amplicons were visualized under ultraviolet light using a transilluminator. The images were captured for photodocumentation (Loccus, Brazil).

Table 1
Sequence, amplicon size, and references for the primers used in this study

Primer	5′-3′ sequence	Amplicon size	Reference
MGPCR/732	GGATCCCATCTCGACCACGACAAAA	732 pb	(Nascimento et al., 1991)
	CTTTCAATCAGTGAGTAACTGATGA		
MGPCR/481	GTAGCAAGTTATAATTTCAGGCAT	481 pb	(Nascimento et al., 2005a)
	CGTGGATATCTTTAGTTCCAGCTGC		
16S	GAAGCAAAATAGTGATATCA	207 pb	(Lauerman et al., 1993)
	GTCGTCTCCGAAGTTAACAA		
*mgc2**	GCTTTGTGTTCTCGGGTGCTA	829 pb	(Ferguson et al., 2005)
	CGGTGGAAAACCAGCTCTTG		

*Sequencing primers.

Genotyping Mycoplasma gallisepticum

To establish relationships among the isolates, the *mgc2* gene of all isolated colonies was amplified and sequenced, following the protocol described by Ferguson et al. (2005). The reaction mixture contained 1× PCR buffer, 0.2 mM dNTPs, 0.2 µM of each specific primer, 1 U Hot Start Taq polymerase (Promega, Brazil), and 5 µL of DNA. The amplicons were purified using the QIAquick PCR purification kit (Qiagen) and sequenced using an ABI 3730 DNA sequencer (Applied Biosystems, USA) at the Fiocruz Sequencing Platform. The evolutionary history was inferred using the Maximum Likelihood method with the Kimura 2-parameter model (Kimura, 1980). Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). In addition to the strains from the present study, sequences from strains detected in other Brazilian states and the most commonly used vaccine strains (MG-F and MG-70) were included in the phylogenetic analyses. The gene encoding the P30 protein, homologous to the *M. gallisepticum mgc2* gene product, from *M. pneumoniae* was used as an outgroup.

Results

Of the 172 samples collected, PCR analysis detected MG in 43 samples (25.00%) and MS in 123 samples (71.51%). Table 2 details the presence of MG and MS in the growing and production phases for each poultry farm. None of the flocks tested positive for MS by culture, while four flocks tested positive for MG.

Table 2
Frequency of detection of *Mycoplasma gallisepticum* and *M. synoviae* by PCR in commercial laying hens from Sao Paulo state

Poultry farm	Flock age (in weeks)	MG detection (%)	MS detection (%)
A	35	0/13 (0%)	9/13 (69.23%)
B	9	0/11 (0%)	9/11 (81.82%)
	17	2/11 (18.18%)	2/11 (18.18%)
	45	1/11 (9.09%)	6/11 (54.55%)
	24	2/11 (18.18%)	5/11 (45.45%)
C	10	0/11 (0%)	11/11 (100%)
	78	0/11 (0%)	4/11 (36.36%)
	29	10/11 (90.91%)	9/11 (81.82%)
	31	0/11 (0%)	9/11 (81.82%)
D	23	3/11 (27.27%)	4/11 (45.45%)
	38	9/12 (75.00%)	12/12 (100%)
E	27	6/12 (50.00%)	12/12 (100%)
F	47	0/11 (0%)	8/12 (66.67%)
	Unknown	11/12 (91.67%)	9/12 (83.33%)
	30	0/12 (0%)	12/12 (100%)
	Total	44/172 (25.58%)	123/172 (71.51%)

Phylogenetic analysis of the *Mycoplasma gallisepticum* strains revealed distinct clustering patterns, with our isolates forming closely related groups (Figure 1) Reference strains, such as *M. gallisepticum* MG-70 and strain F, were positioned separately from the more recent isolates. The branch lengths indicate varying degrees of genetic divergence among the strains, reflecting their evolutionary relationships.

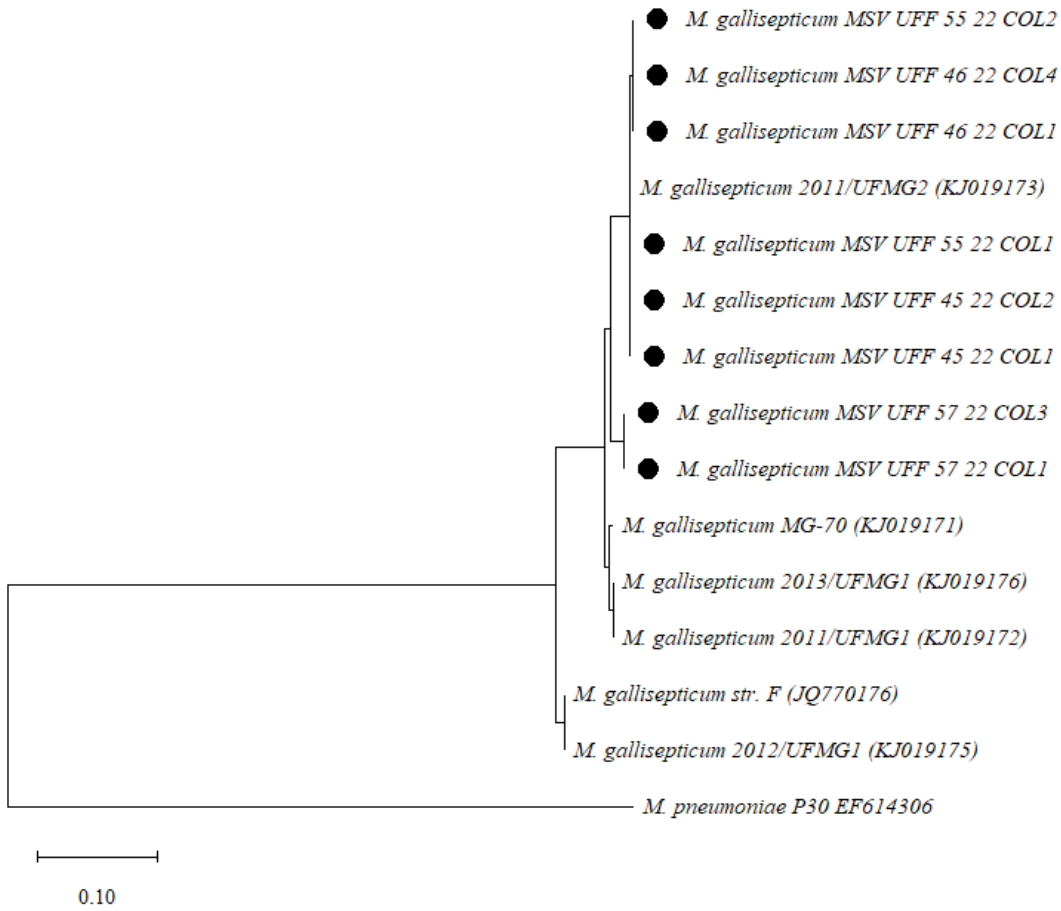

Figure 1. Phylogenetic tree of Brazilian MG and vaccine strains based on the alignment of the partial *mgc2* gene constructed with the Neighbor-joining maximum-likelihood (NJML) method using Mega X software. The black dot indicates strains for this study.

Discussion

The diagnosis of mycoplasmosis can be established through a combination of zootechnical data analysis, clinical signs, pathological lesion interpretation, serological tests, and the detection of mycoplasmas by isolation and/or polymerase chain reaction (PCR) (Nascimento et al., 2020). Isolation and identification of the organism constitute the "gold standard" for diagnosing mycoplasma infections (Beylefeld et al., 2018) and require specific culture media, with Frey medium being commonly used for avian mycoplasma cultivation. Despite the satisfactory growth of MG and MS in Frey medium, these species grow slowly and can be easily outcompeted by faster-growing mycoplasmas (Hong et al., 2004; Khalifa et al., 2013), such as *M. gallinaceum.* MS and MG colonies typically appear on agar medium within 3 to 21 days after inoculation. However, non-pathogenic species can develop colonies within 24 h, compromising the isolation of pathogenic avian mycoplasmas (Fialho et al., 2023).

PCR analysis detected a high frequency of MS in laying hens. Studies have shown that MS infection is widespread in Brazil, with some regions reporting prevalence rates as high as 70% (Buim et al., 2009; Santos et al., 2021; Silva et al., 2020; Teixeira et al., 2015). The absence of routine vaccination against MS in Brazilian farms might explain the high detection rates observed.

Although MG vaccination is a common practice in Brazil, particularly with live vaccines such as MG-70 and MG-F, approximately 25% of the hens in this study tested positive for MG. This detection rate is similar to that reported by Silva et al. (2020) in the same region but higher than those found in other studies (Buim et al., 2009; Santos et al., 2021; Teixeira et al., 2015). The high density of laying hens in the study area may facilitate pathogen transmission among farms, necessitating strict biosecurity measures to prevent pathogen entry (Silva et al., 2021).

The subclinical progression of many MS infections allows mycoplasmas, particularly MS, to persist on farms. Consequently, these bacteria can remain in the farm environment and potentially spread to other animals without causing observable symptoms. This subclinical progression of MS has significant implications for farm management, as it signifies that despite the absence of visible disease, the bacteria may still be present and impact overall farm health and productivity. Additionally, there is significant concern regarding mycoplasmas' ability to induce immunosuppression and exacerbate the pathogenic effects of other infectious agents (Silva et al., 2021).

Studies have shown that *mgc2*-based genotyping provides a more comprehensive understanding of *M. gallisepticum's* genetic diversity. Limsatanun et al. (2022) used a combination of *gapA*- and *mgc2*-based genotyping to differentiate *M. gallisepticum* strains isolated from various regions of Thailand. Their results demonstrated that this method is highly sensitive and specific, offering valuable insights into the genetic diversity of *M. gallisepticum.* Phylogenetic analysis revealed distinct clustering patterns among the *M. gallisepticum* strains, highlighting genetic divergence and potential epidemiological linkages. Several isolates, particularly those from our study formed closely related clades, suggesting a common evolutionary origin or recent transmission events. Notably, strains collected in different years (e.g., 2011/UFMG1, 2012/UFMG1, and 2013/UFMG1) exhibited genetic variation, indicating ongoing evolutionary changes within the population. The reference strains, including *M. gallisepticum* MG-70 and strain F, clustered separately from the more recent isolates, reinforcing their established genetic distinction. The low degree of similarity among these strains may be explained by the notably high mutation rate of mycoplasmas, which results in significant intra-specific variation and the emergence of multiple distinct strains (Matucci et al., 2020).

In our study, PCR analysis detected a high prevalence of MG and MS; however, only MG strains were successfully isolated through culture. *mgc2* genotyping revealed a high degree of similarity between the detected strains and a low degree of similarity between the vaccine strains and strains from other regions.

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Conflict of Interest Statement

The authors declare no competing interests.

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