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Original Article

Impairment in the Isolation of Mycoplasma synoviae in Mixed Infection with Mycoplasma gallinaceum in Laying Hens

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ABSTRACT

Avian mycoplasmosis is a disease of significant economic impact, potentially leading to restrictions on the international trade of poultry products. *Mycoplasma synoviae* (MS) and *Mycoplasma gallisepticum* (MG) are well-established as pathogenic, while *Mycoplasma gallinaceum* (Mgc) is considered commensal. Nevertheless, the latter can exacerbate clinical conditions in cases of co-infections with other pathogens. Due to the slow growth rates of MS and MG, they are susceptible to being outpaced by fast growing mycoplasmas, thereby complicating cultivation, and potentially yielding false-negative results. Thus, this study aimed to report a mixed infection of Mgc and MS in commercial laying hens and the compromised isolation of MS caused by this non-pathogenic mycoplasma. Twenty tracheal samples from chickens were collected, subjected to PCR, and cultured in a modified Frey's medium. Samples displaying colonies compatible with mycoplasmas underwent three passages to obtain pure cultures. PCR-positive samples for the *Mycoplasma* spp., and negative ones for MS and MG, were subsequently subjected to DNA sequencing. All clinical samples tested via PCR were positive for MS (20/20) and negative for MG (0/20). During isolation, only five samples exhibited colonies with characteristic mycoplasma growth. However, PCR analysis of these cultures produced negative results for MS and MG, but were positive for *Mycoplasma* spp. DNA sequencing confirmed that all isolated strains were Mgc. Mgc displayed rapid growth, posing challenges to obtaining MS strains for isolation. The coexistence of MS and Mgc in the respiratory tract of commercial laying hens has the potential of inducing a synergistic effect on respiratory manifestations.

INTRODUCTION

The significance of the poultry sector in Brazilian agribusiness has made the health status of poultry flocks a constant concern, due to its potential to act as a barrier to the commercialization of poultry and poultry products. Respiratory diseases such as avian mycoplasmosis have the most substantial impact on the poultry industry, posing a significant economic threat, including restrictions on poultry product exports. The consequences of avian mycoplasmosis encompass challenges in the rearing phase, characterized by increased rates of chick culling and mortality. In the production phase, there is a marked decline in both the quantity and the internal/external quality of commercial poultry. (Nascimento *et al*., 2020).

Currently, there are approximately 25 characterized species of avian mycoplasmas (Ferguson-Noel *et al*., 2020); while some are pathogenic, the majority is classified as non-pathogenic. *Mycoplasma synoviae* (MS) and *Mycoplasma gallisepticum* (MG) are well recognized pathogenic species of great concern to the poultry industry due their ability to cause

visible or subclinical diseases in poultry (Nascimento, 2020). *Mycoplasma gallinaceum* (Mgc), is a non-pathogenic species frequently isolated in the upper respiratory tract of commercial poultry (Bradbury *et al*., 2001). Although not causing disease on its own, Mgc can enhance the pathogenicity of other respiratory pathogens in cases of co-infection (Adeyemi *et al*., 2018).

The diagnosis of mycoplasmosis includes the analysis of epidemiological data, observation of clinical signs, interpretation of anatomopathological lesions, serological tests, and the detection of mycoplasmas through isolation and/or PCR (Nascimento *et al*., 2020). Mycoplasma isolation and identification are considered the "gold standard" for diagnosis (Beylefeld *et al*., 2018) and require specific media, such as Frey's medium. It is important to note that MS and MG grow slowly in this medium and can easily be outcompeted by other fast-growing mycoplasma species, such as Mgc (Kleven, 2008). MS and MG colonies can take anywhere from 3 to 21 days to form on agar, while non-pathogenic species can develop colonies within 24 hours, complicating the isolation of pathogenic avian mycoplasmas (Ferguson-Noel *et al*., 2020; Nascimento *et al*., 2020).

The aim of this study was to report the compromised isolation of MS due to MgC coinfection in commercial laying hens.

MATERIALS AND METHODS

Twenty tracheal samples from laying hens in two different production batches were collected using swabs. These samples were stored in tubes containing modified liquid Frey medium (Pereira *et al*., 2022) and sent to the Avian Health Laboratory at the Federal Fluminense University for processing. 200 µl from each sample were aliquoted for mycoplasma detection by culture, and 500 µl were allocated for PCR.

Culturing was conducted using both liquid and solid modified Frey medium. Specimens in liquid medium that exhibited yellowing due to medium acidification resulting from bacterial growth were plated on solid medium to isolate colonies compatible with mycoplasmas (mamillary or fried-egg-like appearance). Isolated colonies underwent three passages on solid and liquid media to obtain pure colonies. At each passage, aliquots were subjected to PCR for genus and species identification as describe above.

For PCR, DNA extraction was performed using the phenol-chloroform method adapted from Sambrook & Russell (2006). All samples, along with positive controls, were quantified and assessed for purity using a Biodrop Touch® spectrophotometer (Biochrom). Extracted samples were then subjected to PCR for the *Mycoplasma* genus (van Kuppeveld *et al*., 1994) MS, and Nested-PCR for MG according to Lauerman *et al*. (1993) and Nascimento (1992, 2005), respectively. Each reaction was carried out with 100 ng of genomic DNA.

PCR amplicons were applied to a 1.5% agarose gel, submerged in 1X Tris-Acetate-EDTA buffer (TAE), and subjected to electrophoresis at 94 Volts for 40 minutes. The amplified bands were stained with 0.5% ethidium bromide and visualized under ultraviolet light using a transilluminator.

Amplicons that were positive for the *Mycoplasma* genus in PCR and negative for MS and MG were purified using a QIAquick PCR purification kit (Qiagen, USA), and sequenced with an ABI 3730 DNA sequencer (Applied Biosystems) using the RPT/Fiocruz Sequencing Platform. Evolutionary history was inferred using the Maximum Likelihood method and the Kimura-2 parameter model. Evolutionary analyses were conducted using the MEGA X software.

RESULTS AND DISCUSSION

All tracheal material samples collected and initially tested by PCR were positive for MS (20/20) and negative for MG (0/20). However, after cultivation and isolation, only 5 samples exhibited colonies with typical mycoplasma growth. When these samples underwent PCR confirmation, they yielded negative results for MS and MG, but tested positive for Mycoplasma spp. Sequencing of these samples revealed a likelihood pattern for Mgc.

Out of these 5 sequenced samples, only 3 yielded results with the necessary scores for phylogenetic analyses. The sequences obtained in this study have been deposited in the GenBank database with accession numbers OR553113 to OR553115. It was evident that the strains initially identified as *Mycoplasma* spp. by PCR were indeed Mgc and displayed a high degree of similarity among themselves (Figure 1).

These findings underscore the importance of employing molecular methods such as PCR and sequencing for the identification of *Mycoplasma* spp. However, isolation still constitutes a crucial diagnostic method, as obtaining pure cultures is necessary for strain characterization during outbreaks (Boettger & Dohms, 2006).

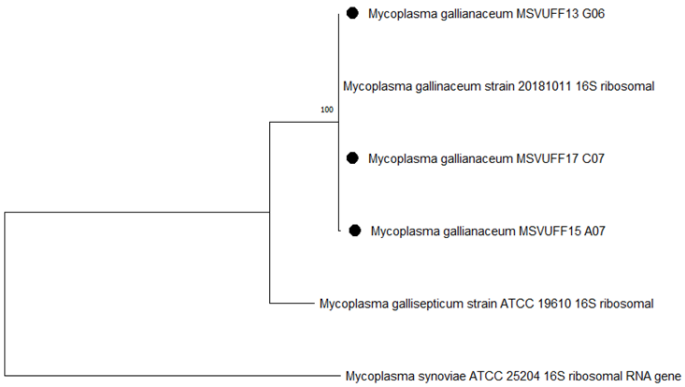

Figure 1 – Phylogenetic Tree of *Mycoplasma gallinaceum* in commercial laying hens.

In this study, difficulties were encountered in obtaining pure MS cultures due to the rapid growth of Mgc. A similar situation has been previously reported by Beylefeld *et al*. (2018) and Abed *et al*. (2021), who found that non-pathogenic mycoplasma species were more frequently isolated than pathogenic ones in avian mycoplasma isolates. It is worth noting that despite being considered non-pathogenic, Mgc can colonize various sites within chickens (Santos *et al*., 2018), and can exacerbate clinical conditions in co-infections with MS; other respiratory agents (Yagihashi *et al*., 1993 and Adeyemi *et al*., 2017). Mgc strains are often isolated during attempts to culture pathogenic mycoplasmas and have been found in the upper respiratory tract and oviducts of sick birds (Bradbury *et al*., 2001). Therefore, the rapid growth of Mgc hinders the isolation of pathogenic mycoplasmas, but its commensal nature and consequent lesser significance have led to limited research on its role in poultry, requiring further investigations into this microorganism.

CONCLUSION

Due to its rapid growth, the presence of Mgc caused difficulty in isolating pathogenic strains of avian mycoplasmas, and confirmed its association with MS in the respiratory tract of commercial laying hens. All Mgc strains found in this study exhibited a high degree of similarity in the phylogenetic analysis.

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