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SCIENTIFIC ARTICLE
Animal Epidemiology

Occurrence of *Mycoplasma gallisepticum* and avian metapneumovirus in commercial broiler flocks from the Southeast and Midwest regions of Brazil

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ABSTRACT

Respiratory tract infections in poultry have become serious problems with negative consequences for poultry production. Among these diseases, mycoplasmosis and avian pneumoviruses stand out, which, despite being relevant in poultry health, have not been systematically investigated in Brazil, especially regarding the interaction between these agents or the occurrence of co-infection in broiler chickens. The present study investigated the occurrence of infection by *Mycoplasma gallisepticum* (MG) and subtypes A and B of avian metapneumovirus (aMPV) in broiler chickens from commercial poultry flocks kept on farms with high biosecurity standards and located in the Southeast and Midwest regions of Brazil. Polymerase chain reaction (PCR) and reverse transcription nested-PCR techniques were applied to detect and/or identify, respectively, MG and aMPV in samples of nasal and tracheal swabs collected between 2017 and 2018, from 87 batches of broiler chickens from 15 commercial production farms that showed respiratory clinical signs. Two out of 87 batches sampled (2.3%) were positive for MG, while none of them were positive for aMPV. The low or no incidence of these pathogens can be explained by the adoption of increasingly effective health control measures for these agents on farms with high biosecurity standards. In addition, there are clues that other bacterial and viral infectious agents may be involved in the etiology of respiratory problems of these broiler chickens, which showed clinical signs of respiratory diseases upon sample collection.

Keywords: broiler production; PCR; respiratory diseases; RT-Nested-PCR.

INTRODUCTION

Brazil is the largest exporter and the second largest producer of broiler meat in the world, according to the Brazilian Animal Protein Association (ABPA, 2021).

Pathogens that affect the respiratory tract of birds, mainly chickens used in poultry production (*Gallus gallus*), can affect a large number of animals, and, depending on the agent involved, high mortality may also occur. Furthermore, infected chickens present, in addition to respiratory syndromes, reduced growth and/or production capacity, leading to significant economic losses (Jones, 2010).

The pathogen *Mycoplasma galissecticum* (MG) causes an important acute infectious disease in chickens and turkeys, especially in young chicks, which induces respiratory diseases (OIE, 2021). This bacterium and *Mycoplasma synoviae* are the most pathogenic of the *Mycoplasma genus* (Kleven, 2008), and, due to the production losses induced, they hold high economic importance for poultry production, occurring in all regions of the world (Kleven, 2008; OIE, 2021).

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The practicality and accessibility of molecular diagnostic techniques have increased their use for the detection of MG in biological samples from chickens suspected of infection by this pathogen. Different procedures for the polymerase chain reaction (PCR) technique and oligonucleotide primers have been described, depending on the target gene for amplification in this technique (García et al., 2005; Raviv et al., 2007; Hossam et al., 2016; Wu et al., 2019).

In Brazil, several studies in the last 20 years were carried out to determine the occurrence or prevalence of MG in chickens from commercial poultry farms of layers, broilers, and breeders raised in different regions. For this, serological methods, as well as molecular techniques, were used. As a result, the prevalence of MG infection observed in broiler chickens by molecular techniques ranged from 2 (in the state of Pernambuco) to 3.8% (in the states of Paraná and São Paulo) (Buim et al., 2009; Barros et al., 2014), and higher percentages of prevalence were found, ranging from 20 (in the state of Rio Grande do Sul) to 32.25% (in the state of Goiás) (Minharro et al., 2001; Machado et al., 2012) in broilers affected by more prominent respiratory lesions. In addition, a variation from 9.28 to 11.37% in prevalence was observed in studies using serodiagnostic methods to detect MG infection (Teixeira et al., 2015). In laying chickens, prevalence ranges from 2 to 50% using molecular techniques (Buim et al., 2009; Casagrande et al., 2014; Teixeira et al., 2015; Silva et al., 2021) and variations from 42.92 to 100% were reported, when serological methods were used as a form of indirect diagnosis (Casagrande et al., 2014; Teixeira et al., 2015).

Thus, it appears that there is a limited number of studies on the occurrence of MG infection in broiler chickens in Brazil, and there is a predominance of data obtained from studies of this pathogen in laying hens and "backyard" birds.

Avian metapneumoviruses (aMPV) are characterized by causing an acute and highly contagious infection of the upper respiratory tract of turkeys and chickens. Although the role of aMPV as a primary pathogen is less evident, there are several indications that it is one of the factors involved in the etiology of a severe form of sinusitis in chickens, known as swollen head syndrome (Cook, 2000).

The aMPV strains have been classified into four subtypes–A, B, C, and D–, based on analysis of the nucleotide sequence of the target cell membrane receptor-binding glycoprotein gene (glycoprotein G) (Cook; Cavanagh, 2002; OIE, 2022). In Brazil, only subtypes A and B of aMPV have been identified (Chacón et al., 2011; Felippe et al., 2011).

Along with this, the diagnosis of aMPV infections in turkeys and chickens can be made using molecular techniques, such as conventional, or reverse transcription (RT) PCR and RT-nested-PCR, or serological techniques, such as the serum neutralization test and enzyme-linked immunosorbent assay (ELISA). This last method is the most used in the immunodiagnosis of aMPV infection (Cook, 2000; Ferreira et al., 2009).

Some studies were carried out in Brazil, aiming to determine the occurrence or prevalence of aMPV in chickens from commercial poultry farms in different regions of Brazil, between the years 2004 and 2011. The main results of these studies revealed a variation in the occurrence of aMPV infection from 10.4 to 18.3%, using serological or molecular methods to detect infection by this agent in broiler chickens (Boaro et al., 2004; Peres et al., 2006; Chacón et al., 2011; Felippe et al., 2011).

Due to the lack of more updated data on the occurrence of infections by MG and aMPV in highly technified broiler farms in Brazil, the present study aimed to survey the occurrence of infection by MG and subtypes A and B of avian aMPV in broiler chickens from poultry flocks on commercial farms in the Southeast and Midwest regions of Brazil.

MATERIAL AND METHODS

Mycoplasma gallisepticum and avian metapneumovirus reference strains

Attenuated vaccine strain RTV8544 (Nobilis RTV8544; Laboratório Merck Sharp and Dohme, São Paulo, Brazil) and the attenuated vaccine strain PL21 (Nemovac; Laboratório Merial, São Paulo, Brazil) were applied for the molecular analysis of aMPV of subtypes A and B of the aMPV, respectively. The attenuated vaccine strain of MG (Merck Sharp & Dohme, São Paulo, Brazil) was used for the molecular analysis of MG.

Species sampled, study area, and sample collection

Nasal and tracheal swabs were collected, between 2017 and 2018, from 87 flocks of 15 commercial broiler chicken farms highly technified and located in the Southeast regions (states of São Paulo and Minas Gerais) and Midwest (states of Mato Grosso and Goiás) of Brazil. These broiler chickens were presenting respiratory clinical signs, and no one received vaccines against MG or aMPV. Therefore, 10 samples of nasal and tracheal swabs were collected from each of the flocks investigated.

The current trial was approved by the Ethics Committee on the Use of Animals of Universidade Estadual Paulista "Júlio de Mesquita Filho", Jaboticabal, SP, Brazil (Protocol no. 008022/18). After collecting, 10 swabs were pooled for each flock sampled and conditioned in Eagle's minimum essential medium or in tryptose phosphate broth and then stored at -80°C for subsequent extraction of bacterial DNA and viral RNA.

DNA extraction for molecular analysis of *Mycoplasma gallisepticum* from swab samples

The DNA from swab samples was extracted using the QIAamp DNA Mini Kit (QIAGEN), according to the manufacturer's instructions. Finally, the DNA samples were measured according to absorbance in a spectrophotometer (Nanodrop, Thermo Scientific) at wavelengths of 230, 260, and 289 nm, and the ratios 260/280 and 260/230, as well as their concentrations were determined. After that, they were stored at -20°C for subsequent PCR assays.

RNA extraction for molecular analysis of avian metapneumovirus from swab samples

The RNA from nasal and tracheal swab samples was extracted using the Trizol RNA extraction protocol, as described by the manufacturer (Invitrogen, United States of America). At the end, the extracted RNA samples were measured for absorbance in a spectrophotometer (Nanodrop, Thermo Scientific) at wavelengths of 230, 260, and 289 nm, and the ratios 260/280 and 260/230, as well as their concentrations, were determined. Afterward, these samples were subjected to the reverse transcription (RT) reaction to generate cDNA (Cavanagh et al., 1999).

Molecular detection of *Mycoplasma gallisepticum* and avian metapneumovirus

Conventional polymerase chain reaction for the detection of *Mycoplasma gallisepticum* focused on the mgc2 gene

The primer oligonucleotides used in this study for the detection of MG were: *mgc2* 2F 5' CGCAATTTGGTCCTAATCCCCAACA 3', and *mgc2* 2R 3' TAAACCCACCTCCAGCTTTATTTCC 5', which amplify a fragment of 236-302 bp (García et al., 2005). The mixture for carrying out the PCR technique was made with some modifications according to the methodology described by García et al. (2005) for the detection of the *mgc2* gene of MG. The mixture was prepared in a final volume of 25 µL, containing 2.5 µL of 10x concentrated buffer, 0.5 µL of TaqPolymerase (5 U/µL), 1 µL of 400 mM dNTPs, 0.75 µL of MgCl2, 16.75 µL of nuclease-free water (Promega Corp.), 0.5 µL (25 pmol/µL) of each of the oligonucleotide primers, and 5 µL of template DNA extracted from the biological samples that were analyzed.

The conditions for carrying out this reaction PCR were: a first cycle of 5 min at 94°C, followed by another 35 cycles of 94°C of incubation for 1 min, 58°C for 1 min, and then 72°C for 2 min, come after by a final extension at 72°C for 7 min. The MG 6/85 vaccine strain (Merck Sharp & Dohme) was utilized as a positive control for DNA extraction and PCR. Furthermore, a negative control consisting of nuclease-free water was also used in each PCR.

Reversion transcription nested-polymerase chain reaction for the detection and identification of avian metapneumovirus subtypes A and B

RNA preparations extracted from swab samples collected from broiler chickens were subjected to the RT-Nested-PCR technique, using primer oligonucleotides designed by Juhasz; Easton (1994) and the methodology described by Cavanagh et al. (1999), with modifications, to detect and differentiate subtypes A and B of aMPV.

The RT reaction mixture was prepared as follows: 1 µL of random primer and 4 µL of diethyl pyrocarbonate (DEPC) water were added to the final volume of 5 µL, adding 5 µL of the extracted RNA sample. Cycling was made at 70°C for 5 min and 4°C for 1 min. In addition, a second step was taken so that the transcription could be carried out. In this step, the mix contained: 5 µL of buffer (Buffer 5x), 1.25 µL of 2'-deoxynucleoside 5'-triphosphates (dNTPs) (100 mM), 1 µL of RNAse OUT, 1 µL of the M-MMLV enzyme, and 6.75 µL of DEPC water, totaling the volume of 15 µL, with 10 µL added of the product from the first mix mentioned.

The RT incubation comprised a first step at 37°C for 60 min, followed by another step at 38°C for 10 min, and then maintained at 4°C. The PCR mixture was prepared in a final volume of 25 µL, containing 2.5 µL of 10x concentrated buffer, 0.25 µL of Taq DNA Polymerase (5 U/µL) (Invitrogen), 0.5 µL of dNTPs at 400 mM, 0.75 µL of MgCl2, 16.5 µL of

nuclease-free water (Invitrogen), 1 µL (10 pmol/µL) of each of the primer oligonucleotides, and 5 µL of the template cDNA extracted from the biological samples that were analyzed.

The second PCR (RT-Nested-PCR) was performed separately with pairs of primers internal to the DNA sequence amplified in the first PCR in order to amplify two regions of the aMPV G gene; one of them with 268 bp and the other one with 361 bp, which, respectively, correspond to subtypes A or B of this virus. The first and nested PCRs were performed using the following cycling profiles: denaturation (94°C, 1 min), annealing (50°C 1.5 min), extension (72°C, 2 min), for 30 cycles, and a final extension at 72°C for 7 min. Preparation of cDNA obtained at RT from RNAs extracted from samples of the RTV (Nobilis RTV 8544, Merck Sharp and Dohme) and PL21 (Nemovac, MERIAL) vaccine strains were used as positive controls for aMPV subtypes A and B. A negative control consisting of nuclease-free water was also included in each Nested-PCR technique protocol.

Agarose gel electrophoresis

Subsequently to the PCR and RT-Nested-PCR reactions, the amplified products were analyzed using the horizontal electrophoresis technique, at the voltage of 60V for approximately 2 hours, in a 1.5% agarose gel and stained with SYBR™ Gold Nucleic Acid Gel Stain (InvitrogenTM) (10,000X concentrate in DMSO). The "1kb DNA plus Ladder" marker (Invitrogen) was used as a molecular weight standard for electrophoresis of the PCR products. The gels were visualized using ultraviolet light on a transilluminator (Syngene), photo-documented with a digital camera (Synoptics), and processed using the Gene Link software (Syngene).

RESULTS AND DISCUSSION

Two of the 87 batches of broiler chickens analyzed (2/87; e.g., 2.3%) were positive for MG (Fig. 1). It should be noted that the two sets of swab samples detected as positive for MG were collected from two batches of 23-day-old broilers raised in a farm located in the Southeast region of Brazil.

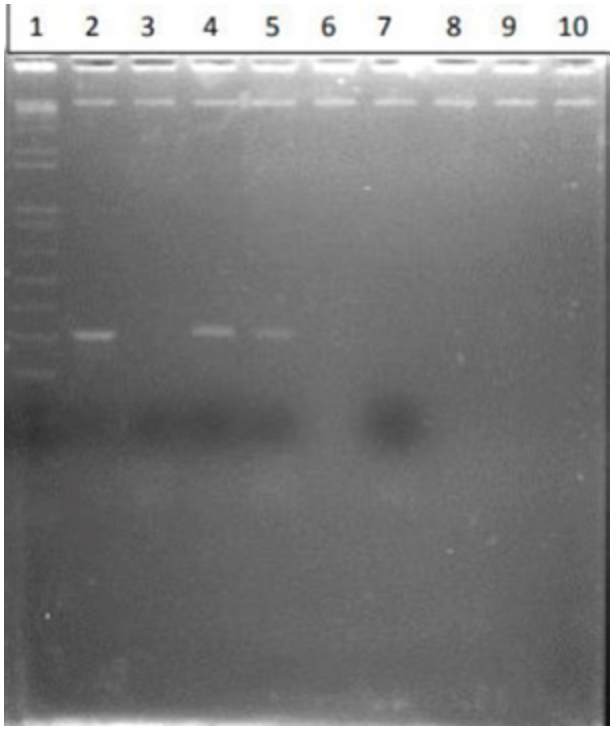

Figure 1. Electropherogram of 1.5% agarose gel as a result of polymerase chain reaction amplification of the *Mycoplasma gallisepticum* (MG) *mgc2* gene in tracheal swab samples from broiler chickens. Lane 1: molecular marker 1 kb DNA plus ladder. Lane 2: positive control for MG. Lane 3: negative tracheal swab sample. Lanes 4 and 5: positive tracheal swab samples. Lane 6: negative control for MG.
Source: Elaborated by the authors.

Regarding aMPV, none of the nasal and tracheal swab samples from the 87 broiler batches analyzed revealed positivity for the presence of subtypes A or B of this pathogen.

The percentage of MG occurrence (2.3%) found in this study in broiler chickens was slightly lower than the 3.8% (5/129) found by Buim et al. (2009) after PCR analysis of a set of 129 samples of tracheal swabs collected between 2001 and 2004 from the same type of chickens in the states of Paraná, Pernambuco, and São Paulo. According to this latest investigation, the percentage of positivity found for the presence of MG in all chickens sampled from commercial poultry farms, including broilers, laying hens, and breeders, was 2% (21/1,046), which is similar to the percentage of positivity that was observed in the present study.

Additionally, Barros et al. (2014) investigated the occurrence of MG and *Mycoplasma synoviae* in commercial broilers and layers raised on 24 farms in the state of Pernambuco and observed that only one sample (4.17%) was positive for MG and seven samples (29.17%) were positive for *M. synoviae*.

It is interesting that another study by Teixeira et al. (2015) did not report any positive results in the PCR technique for the presence of MG in tracheal swab samples collected from 127 samples distributed in 25 flocks of commercial broiler chicken farms in the state of Rio de Janeiro. However, in that same study, a 5% positivity for MG was detected in a set of 115 samples of tracheal swabs from laying hens kept in 20 flocks in the same state of Brazil.

In addition, Santos et al. (2021) found the occurrence of MG in a set of 604 tracheal samples collected from broiler chickens in a slaughterhouse in the Distrito Federal (Brazil) in 2017, detecting five positive samples for this microorganism using the PCR technique, making a positivity of 0.8%, which is lower than the percentage detected in the present study.

In general, lower percentages were found in the current study for positive samples for MG compared to other studies carried out in Brazil in the periods ranging from 10 to 22 years ago, as occurred in data surveys carried out by Minharro et al. (2001) and Machado et al. (2012), which detected 32.25 and 20% of positive samples for MG, respectively. However, it should be noticed that these studies have analyzed samples from broiler chickens presenting marked aerosacculitis detected at slaughter, in the states of Goiás (Minharro et al., 2001) and Rio Grande do Sul (Machado et al., 2012), and using the PCR technique with another pair of primers.

Regarding the occurrence of infection by aMPV, no positive samples for this viral pathogen were detected in this study, which contrasts with findings from 10 to 15 years ago, that determined the occurrence of this pathogen in Brazil using different protocols of molecular techniques and primer sets, regarding those used here. Thus, Chacón et al. (2011) analyzed using RT-Nested-PCR techniques a set of 228 samples collected between 2004 and 2008 from broilers, layers, and breeders with respiratory and reproductive disorders and kept in vaccinated and unvaccinated commercial farms located in six geographic regions of Brazil, demonstrating the presence of subtypes A and B of aMPV in six and nine samples of broilers, respectively. Moreover, Felippe et al. (2011) in an epidemiological survey of the presence of aMPV in wild and synanthropic birds and in broiler chicken farms, in the period between 2008 and 2009, reported 15 (12.9%) positive samples for subtypes A, or B of aMPV collected from broiler chickens with clinical signs of swollen head syndrome from various regions of Brazil.

The interpretation of the reduced occurrence of MG and the absence of aMPV infections in our study for broilers from poultry flocks located in the Southeast region (states of São Paulo and Minas Gerais) and Midwest region (states of Mato Grosso and Goiás) of Brazil may be justified by the improvement that the control of these pathogens underwent in relation to the period of the previous surveys, especially when considering that the samples in the present study were obtained in 2017 and 2018. Another possibility for this result is the fact that there is an additional difficulty in detecting the presence of aMPV in birds after the early acute phase of the infection (Cook; Cavanagh, 2002). Regardless, these respiratory pathogens are an important risk for poultry production, since there are potential challenges of MG infection for broiler chicken farms, as there is a high prevalence of infection by this pathogen in "backyard" chickens, as revealed by the studies of Buchala et al. (2006) and Batista et al. (2020), as well as aMPV infection was detected in wild birds in Brazil (Felippe et al., 2011).

It should also be considered the fact that the respiratory clinical manifestations observed in the broilers sampled in this study may have an etiology caused by other bacterial and/or viral infectious agents that can also affect broiler chickens, such as infectious bronchitis virus, avian pathogenic *Escherichia coli*, *Ornithobacterium rhinotracheale*, *Avibacterium paragallinarum*, and *Pasteurella multocida*, among other avian respiratory pathogens (Silva et al., 2021).

CONCLUSIONS

Broiler chickens from commercial farms located in the Southeast and Midwest regions of Brazil have low or no occurrence of MG and aMPV infections, respectively, probably because these farms are subjected to more strict sanitary measures for

these pathogens. In fact, these poultry farms present a higher level of biosecurity in their management, resulting in more effective prevention of infectious diseases, such as those caused by MG and aMPV agents.

Furthermore, our findings also suggest that other bacterial and viral infectious agents, not investigated in this study, may be involved in the etiology of respiratory problems in these broilers, since the chickens sampled in the present study showed clinical signs of respiratory disease, highlighting the importance of other agents also being studied and monitored in the future, whether through molecular diagnostic or serodiagnostic techniques on these same broiler farms.

AUTHORS' CONTRIBUTIONS

Conceptualization: Montassier, H.J. **Methodology:** Secato, C.T.; Fernando, F.S.; Lopes, P.D.; Pavani, C.; Montassier, M.F.S. **Data Curation:** Secato, C.T. **Writing – Original Draft:** Secato, C.T., Montassier, H.J.

AVAILABILITY OF DATA AND MATERIAL

The data presented in this study are available upon request from the corresponding author.

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CONFLICTS OF INTEREST

Nothing to declare.

ETHICAL APPROVAL

The current trial was approved by the Comissão de Ética no Uso de Animais (CEUA) of UNESP, Jaboticabal, SP, Brazil. Protocol no. 008022/18

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