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Incidence of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in broiler flocks at the Federal District of Brazil and its surrounding areas

Incidência de lotes de frangos afetados por *Mycoplasma gallisepticum* e *Mycoplasma synoviae* no Distrito Federal e Entorno, Brasil

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Highlights

Infection control for MG and MS is essential at all production chain.
Mycoplasma gallisepticum and *M. synoviae* are very important avian pathogens.
Micoplasmoses result in considerable economic losses.

Abstract

Mycoplasma is an important avian pathogen that can cause both respiratory disease and synovitis in birds, resulting in considerable economic losses to the poultry industry worldwide. This study aimed to determine the incidence of *Mycoplasma gallisepticum* and *M. synoviae* in broiler flocks at the Federal District and its surrounding regions using polymerase chain reaction (PCR). All slaughtered lots (57 flocks) were analyzed from July to November in one of the two slaughterhouses at the Federal District with Federal inspection services. Approximately 10 samples of broiler tracheae per slaughtered batch were collected from the evisceration line. The results obtained from the accumulated incidence over the study period were 7.02% and 35.09% for *M. gallisepticum* and *M. synoviae*, respectively. A greater concentration of flocks affected by *M. synoviae* was observed during October. The sample design as well as the PCR technique assisted in detecting both agents in the broiler batches in the first epidemiological study of these two agents in the region.
Key words: Airsaccullitis. Avian respiratory disease. Mycoplasmoses. Broiler.

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Resumo

Os micoplasmas são importantes patógenos aviários, que podem causar doenças respiratórias e sinovites em aves, resultando em consideráveis perdas econômicas para a indústria avícola em todo o mundo. O objetivo deste estudo foi determinar a incidência de *Mycoplasma gallisepticum* e *Mycoplasma synoviae* em lotes de frangos na região do Distrito Federal e Entorno, por meio da reação em cadeia de polimerase (PCR). Todos os lotes abatidos (57 lotes) foram analisados durante os meses de julho a novembro, em um dos dois abatedouros frigoríficos do Distrito Federal com Serviço de Inspeção Federal. Na linha de evisceração foram coletadas cerca de 10 amostras de traqueia de frangos de corte por lote abatido. Os resultados obtidos da incidência acumulada no período de estudo foram de 7.02% para *M. gallisepticum* e 35,09% *M. synoviae.* Uma maior concentração do número de lotes afetados por *M. synoviae* foi observada durante o mês de outubro. O desenho amostral, assim como a técnica de PCR, permitiu a detecção de ambos os agentes nos lotes de frangos de corte analisados, sendo este o primeiro estudo epidemiológico desses dois agentes na região de estudo.

Palavras-chave: Airsacculitis. Avian respiratory disease. Mycoplasmoses. Broiler.

Introduction

Mycoplasmas are prokaryotes devoid of cell walls that belong to the class Mollicutes, phylum Tenericutes, and are taxonomically characterized by the phenotype, serology, and sequence of the 16S rRNA (Brown, Whitcom, & Bradbury, 2007). Approximately 120 different species of Mycoplasma have been identified infecting different organisms; of these, more than 25 species have been isolated from birds (Kleven, 2008). Mycoplasma gallisepticum (MG), M. synoviae (MS), and M. meleagridis (MM) are important pathogens that cause respiratory disease, synovitis, and airsacculitis in commercial birds and turkeys (Kleven, 2008, 2003). Laboratory diagnoses confirm that MG is the most economically significant pathogen in chickens (Bradbury, 2005; Scherer, Scherer, Petry, & Sander, 2011), and the significance of the presence of MS has intensified in several studies worldwide Feberwee, De Vries, & Landam, 2008 ; Landam, Mevius, Veldam, & Feberwee, 2014).

MG strains may vary greatly in virulence and infectivity (Ley, 2003), and synanthropic birds are potential vectors and temporary biological carriers of this pathogen, displaying immune responses different from chickens (Gharaibeh & Hailat, 2011). Infection control for MG and MS is essential at all stages of the industry's production chain to avoid loss of productivity due to decreased production, egg quality, reduced feed efficiency, and high condemnation of carcasses (Kleven, 2008; Nascimento & Pereira, 2009).

The detection of avian mycoplasmosis in Brazil began in the mid-1950s and was reported for the first time by Reis and Nóbrega (1955) at São Paulo, in chickens with airsacculitis and turkeys with infectious sinusitis. Garust and Nóbrega (1956) made the first isolations in birds with chronic respiratory disease. In a study conducted in Rio de Janeiro, mycoplasmosis was observed as the most common disease diagnosed by the Embrapa Ornithopathology Sector, with MG isolated from pecking and unborn eggs, chickens, turkeys, and quails (Mettifogo & Buim, 2009).

The status of Brazil as the second largest producer and world exporter of chicken meat could have been achieved due to the growth of the poultry sector and the quality and maintenance of poultry health, where concerns about hygienic-sanitary products have also increased (Machado et al., 2014). In poultry, MG and MS have a predilection for the mucous membranes lining the upper respiratory and urogenital tracts of chickens and turkeys (Nascimento, 2000), characterizing the respiratory, articular, and urogenital signs (Kleven, 2008).

The National Poultry Health Plan (Portaria Ministerial nº193, 1994) recommends several avenues and laboratory procedures for the effective control of these pathogens in commercial poultry breeding. These procedures include regular serological monitoring in herds using a rapid plate agglutination test and enzyme-linked immunosorbent assay (Kleven, 2008). The success of MG and MS control programs depends on the accuracy and promptness of diagnostic techniques, and polymerase chain reaction (PCR) is the most recommended (Sprygin et al., 2010; Fraga et al., 2013). Research on these two agents has been conducted either through direct or indirect detection in Brazil and worldwide. Although the Federal District and its surrounding regions have increased the production of broilers, representing 1.28% of the country's poultry exports, there are no reports on the detection of these agents (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA], 2019).

PCR has certain advantages over serological techniques as it allows detection and agent typing in clinical samples from asymptomatic animals, treatment using antimicrobials, and detection of agents before the immune response in immunocompromised animals without the need for previous cultivation, particularly when dealing with bacteria that are difficult to grow or that demonstrate slow growth (Moreno, 2009).

Considering the importance of mycoplasmosis in poultry production, related losses in the flocks affected by this disease, limited direct detection in the Midwest region and the state of Goiás, and the absence of studies on MG and MS in broiler flocks in the Federal District and its surrounding regions, this study aimed to estimate the incidence of MG and MS using PCR in broiler flocks slaughtered in the Federal District and its surrounding regions.

Materials and Methods

Origin and sample size

Samples were collected from all flocks of broilers slaughtered at 42 days of age from July to November 2017, in a slaughterhouse with a Federal Inspection Service located in the Federal District. This establishment slaughters chickens from farms located in the Federal District and its surrounding regions. Ten windpipes were randomly collected per day on the evisceration line after the Federal Inspection. Altogether, 604 tracheal samples were collected in 57 days.

Considering that the average size of the flocks in the slaughterhouse locality was 57.000 and the average daily slaughter capacity was 51.000, one lot per day was considered and the independence of the lots was assumed during the observation period. It was considered for sampling delineation the requirement of sanitary vacuum and

disinfection of the sheds and the control of reception of chicks for 1 day.

Sample planning, for the number of samples per flock to be collected, aimed to estimate the minimum number of birds to be examined within each flock to allow their classification as infected or not infected by mycoplasmas. Therefore, the concept of aggregated sensitivity and specificity was used (Dohoo, Martin, & Stryhn, 2003). For calculating the sensitivity and specificity of the diagnostic tests used (PCR-MG and PCR-MS), the values of 90% and 100%, respectively were adopted and a minimum estimate of 30% for the intra-batch prevalence was considered when they were contaminated. The sensitivity and specificity were ≥ 95% for an adequate sample size. Statistical analyzes were performed using the STATA® program (StataCorp, 2011).

Detection of MG and MS using PCR

The samples were stored in a freezer at -80°C (Sanyo®) during the sampling process until PCR was performed. For detecting MG and MS, a 10 μL aliquot of each specimen of scarified trachea was used with the aid of a scalpel. The scarified samples were placed in Eppendorf tubes with 50 μL of Milli-Q water. For extraction, the samples were boiled at 100°C for 10 min. After extraction, DNA was quantified at 10 ng.

PCR was performed using pairs of species-specific oligonucleotides, whose sequences of base pairs were previously selected and validated by Lauerman (1998) for the amplification of fragments of the gene encoding the 16S rRNA. Therefore, for MG, primers B1 "Forward" (5′-CGTGGATATCTTTAGTTCCAGCTGC-3′), and B2 "Reverse" (5′- GTAGCAAGTTATAATTTCCAGGCAT-3′) were used to amplify a fragment of 481 base pairs. For MS, primers MS-f "Forward" (5 'GAGAAGCAAAATAGTGATATCA -3′) and MS-r "Reverse" (5′- CAG TCG TCT CCG AAG TTA ACA A -3′) were used for the amplification of 207 base pair fragments.

The PCR reaction was performed in a total volume of 25 μL of a reaction containing 10 ng of DNA extracted from each isolated sample and 2 μL of template, with a final concentration of 3.0 mM MgCl2. The final concentration of 2.0 mM dNTP was used, with 10 pmoL of each reverse and forward primer and 1 U of Taq polymerase (Invitrogen®). The amplification conditions for MG were based on the Lauerman (1998) protocol with a denaturation temperature of 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and a final amplification cycle at 72°C for 5 min. The amplification conditions for MS were as follows: denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 5 min, and a final amplification cycle at 72°C for 5 min. The PCR products were visualized and photo-documented using a transillumination device (Biorad®).

For the positive control, strains of MS and MG were used, provided by Professor Dr. Maria Lúcia Barreto, from the Laboratory Animals nucleus at Universidade Federal Fluminense, which in turn were inoculated in a modified Frey medium and incubated at 37°C for 48 h at the Food Microbiology Laboratory of FAV/UnB, to verify the viability of the cultivation and quality of the modified Frey medium.

Results and Discussion

Detection of M. gallisepticum and M. synoviae

A total of 28 samples were positive for MS, and five samples were positive for MG. The accumulated incidence of lots affected by MS was 35.9% (95% confidence interval CI: 22.91 - 48.90%) and MG 7.2% (95% CI: 1.95 -17.00%) over the follow-up period. No batch was positive for either agent simultaneously. In the Federal District, two slaughterhouses undergo a Federal inspection service. Therefore, this study on the detection of MG and MS in broilers conducted at one of the slaughterhouses with an approximate monthly housing of 1,150,000 broilers is representative of the region, as the approximate total housing of broilers in December 2018 was 8.388.258 birds (Instituto Brasileiro de Geografia e Estatística [IBGE], 2012).

In an epidemiological study conducted on the prevalence of MG and MS by Haesendonck et al. (2014) in Europe, high prevalence values of 73.2% and 96.4%, respectively were detected in herds and free-range birds, suggesting that this group of birds may act as a potential reservoir for Mycoplasma spp. This result differs from that of the present study regarding the type of sample (free-range birds vs. broilers) as MG were greater in number. This difference may be related to the sanitary control of commercial broiler flocks.

An Egyptian study on broiler flocks aged 57 weeks that showed signs of arthritis detected the presence of MS in 87.5% of joint samples using PCR (Amer, Mekky, & Fedawyh, 2019). In the present study, MS was detected in broilers (35.09%); however, there were no signs of arthritis. Mycoplasmoses can spread through air routes or by direct or indirect contact, with the proximity of the breeding place of other Galliformes, such as backyard birds and free-range chickens, a potential threat to the spread of infections in commercial holdings (Michiels et al., 2016). In a Belgian study using chickens and commercial turkeys, a higher prevalence of MS (12.9%) was observed compared to that of MG (2.7%); both were detected using PCR. These results are similar to those found in this study (Michiels et al., 2016). A study conducted at the Agricultural Research Institute of Ethiopia in broiler chickens also detected MG and MS using PCR in addition to other agents that cause respiratory disease in chickens (Hutton, Bettridge, Christley, Habte, & Ganapathy, 2017).

An Iranian study also detected the presence of MS and MG in samples from broiler flocks using PCR, with MS (100%) in a greater number of samples than MG (25%), similar to the results of the present study (Gharibi, Ghadimipour, & Mayahi, 2018). In Brazil, studies have been conducted to detect agents with results similar to those found in the present study. Machado et al. (2014), in a survey of 40 different flocks of broilers at a slaughterhouse under Federal inspection at Rio Grande do Sul, also detected MG in 37.5% of samples using PCR trachea pools. The difference was only in the sampling method since, in the present study, PCR was performed in individual tracheas.

The results obtained for the detection of MG are also similar to those of a study conducted at Pernambuco, in which 11 broilers and laying hens showed positive PCR and nested PCR for the presence of MG in seven samples (33.33%) (Barros et al., 2014); however, some flocks had clinical respiratory signs, unlike the flocks in the present study as evident in the ante-mortem inspection. In the

state of Goiás, Minharro, Linhares, Andrade, Rocha and Santana (2001) also detected MG in 32.25% and MS in 25.80% swab samples from air sac injuries using PCR in broilers at slaughterhouses located at the municipalities of Itaberaí and Pires do Rio. Fraga et al. (2013) analyzed a multiplex PCR for the detection of both mycoplasmas and obtained 100% specificity and sensitivity in the analysis of MG and 94.7% specificity and 100% sensitivity for MS.

The low prevalence of MG detected in this study could be due to the intense control of the layer breeder flocks against this agent, which has been conducted for several years. This agent is a persistent problem in poultry and efforts have been made to prevent the losses in commercial flocks, particularly in layer breeders using bacterin-based, killed, and live vaccines (Butcher, 2002). The major prevalence of MS is probably observed since the vaccine against this species is not commonly used (Nascimento, Pereira, Nascimento, & Barreto, 2005) compared to that for MG.

The choice of PCR technique as a diagnostic test for the detection of MG and MS in the present study is due to its ability to detect the agent with high specificity and sensitivity (Hofmann, Griot, Chaignat, Perler, & Thür, 2008), without the viable microorganisms in biological samples, making it a reliable method for diagnosing and monitoring animal diseases (Haas & Torres, 2016). It is also difficult to diagnose diseases caused by fastidious microorganisms, that is, those with high nutritional requirements and difficult to grow in vitro, such as certain Mycoplasma and Campylobacter species (Hum, Quinn, Brunner, & On, 1997; Buim, Mettifogo, Timenetsky, Kleven, & Ferreira, 2009).

According to Nascimento and Pereira (2009) and Kleven (2008), the results must be confirmed using more appropriate specialized procedures, such as microbiological isolation and identification, or PCR. However, mycoplasmas are fastidious microorganisms with slow growth, and the development of other commensal bacteria of the same genus (such as M. gallinaceum and M. gallinarum) is common in isolating MG and MS (Kleven, 2008).

In the present study, microbiological cultivation of the detected agents did not verify their viability in PCR-positive samples, however, a study by Muhammad et al. (2018) in Pakistan compared PCR with the culture of MS and MG in samples from the trachea of birds with chronic post-mortem respiratory lesions. They concluded that PCR is a sensitive and reliable tool for the diagnosis of avian mycoplasmosis in field samples being approximately 100% in agreement with the microbial culture and more efficient for detecting than culture (52% vs. 47%). Consequently, PCR has been used as an alternative test for the direct detection of MG and MS in clinical samples and other procedures in the last decade (Rasoulinezhad, Bozorgmehrifard, Hosseine, Sheikhi, & Charkhkar, 2018; Hess, Neubauer, & Hacki, 2007).

In the results observed for each month of batch sample collection for the detection of MS, a progressive increase in positive flocks was observed (Table 1), with the largest number of positive flocks observed in October. This increase may have been influenced by climatic characteristics, with the highest humidity from September to January, which predisposes to respiratory problems and reduces the birds' immunity (Darc Moretti, Dias, Telles, & Balian, 2010). There was no progressive increase in MG, as observed for MS.

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Table 1
Number of flocks of broilers affected by *Mycoplasma synoviae* (MS) and *Mycoplasma gallisepticum (MG)*

FLOCKS	MONTHS				
	July MG/MS	August MG/MS	September MG/MS	October MG/MS	November MG/MS
Negative	10/10	11/11	10/10	5/5	1/1
Positive	0/0	2/1	4/2	13/2	1/0

Minharro et al. (2001), in a study using samples of airsacculitis from condemned carcasses in slaughterhouses located in the state of Goiás, observed a higher frequency of MG (32.25%), MS (25.80%), and *E. coli* (80.64%), and the highest occurrence of airsacculitis was in July and January (Instituto Nacional de Metereologia [INMET], 2019). However, in the present study, positive results were detected in August, September, and October, in agreement with Minharro, Linhares, Andrade, Rocha and Santana (1999) and Onozuka, Hashizume, & Agihara (2009), who considered periods of intense rain with high humidity and high temperatures and times of greater oscillations between maximum and minimum temperatures, in association with the low relative humidity of the air, conditions favorable to the occurrence of the disease.

The results of MG in this study and other studies where the presence of this agent was detected are not in compliance with the legislation, as the mycoplasmoses caused by both agents are part of the list of mandatory notification diseases as per the Organization International Epizootics and the list of animal diseases that must be notified to the Official Veterinary Service (Instrução Normativa N° 50, 2013).

In this study, MG and MS could be detected in broiler flocks at a slaughterhouse located in the Federal District and its surrounding areas because of the sample design. As there are only two slaughterhouses with a Federal Inspection Service in this region, this incidence study can be considered as a representative for determining the presence of both agents in broilers at the Federal District and its surrounding regions.

Conclusion

MG and MS were detected in broiler chickens slaughtered in the Federal District and its surrounding areas. In the analysis conducted between July and November 2017, the accumulated incidence of positive lots for MG and MS was estimated as 7.02% and 35.9%, respectively. The highest number of positive flocks for MS was observed in October, coinciding with the beginning of the period of greatest humidity in the region under study. The use of PCR as a diagnostic aid for MG and MS proved to be a fast and sensitive technique for the detection of these agents, following the scientific literature. Considering the importance of the agents detected in this study and the establishment of the measures

by the Ministry of Agriculture, Livestock, and Supply to control and monitor mycoplasmas in breeding establishments through the National Poultry Health Program, the results of the present study corroborate the need to control the presence of these agents in poultry establishments.

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