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Original Article

Detection of Mycoplasma Synoviae and Other Pathogens in Laying Hens with Respiratory Signs in the Rearing and Production Phases

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ABSTRACT

The present study aimed to investigate the occurrence of *Mycoplasma synoviae* (MS), *M. gallisepticum* (MG), *Ornitobacterium rhinotracheale* (OR), *Avibacterium paragallinarum* (AP), *Pasteurella multocida* (PM) and Infectious Bronchitis Virus (IBV) in laying hens with respiratory clinical signs in two phases of production. 140 tracheal swabs and 140 blood samples were collected from laying hens in the rearing and production phases, the chickens belonged to six farms (A-F) located in the state of São Paulo, Brazil. The samples were analyzed by PCR for MG, MS, OR, AP, PM and IBV and by ELISA for MG and MS. The highest frequencies observed by PCR were for MS at farms B and C with 95 and 100% positivity, followed by MG at farms D and E with 35% and 65%, IBV with 35% at farm F and ORT with 15% at farm A. All flocks were positive for MG and MS in serology. Although MG and IBV have been detected, this can be explained by the vaccination protocols, since live attenuated vaccines are widely used for immunization against these pathogens. It was also possible to detect OR and AP thorugh PCR in some flocks. The occurrence of several etiological agents that cause respiratory diseases in laying hens was confirmed by PCR and serology, with MS being the most prevalent and being present in all farms studied.

INTRODUCTION

Respiratory diseases in birds are among the ones with the most impact on production cost and volume (Huton *et al.*, 2017). The synergism among different pathogens can lead to a serious respiratory disease, which substantially reduces the zootechnical potential (not only in laying hens, but also in other poultry species), hinders treatment and control and, consequently, causes great economic losses. The state of São Paulo is responsible for 32.97% of the national egg production (ABPA, 2020), but little is known about which respiratory pathogens are involved in the etiology of the respiratory disease frequently affecting laying hens. Some studies have already been carried out to verify the presence of *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS), and a high prevalence of both was reported in laying hen production (Teixeira *et al.* 2015; Sid *et al.*, 2015). These two are considered the most important agents of diseases in the respiratory system, also affecting hens' reproductive system and joints (Nascimento *et al.*, 2005). Additionally, *Ornitobacterium rhinotracheale* (OR), *Avibacterium paragallinarum* (AP), *Pasteurella multocida (*PM) and infectious bronchitis virus (IBV) can interact synergistically, increasing the severity and duration of respiratory diseases (Sid *et al.*, 2015; Hutton *et al.*, 2017; Jordan, 2017). Polymerase chain reaction (PCR) and serological survey, in association with zootechnical data and clinical signs, can be useful tools in the diagnosis and epidemiological studies

of these diseases (Stipkovits & Kempf 1996; Pang *et al.*, 2002). Research to determine the etiology, prevalence, and degree of involvement of each microorganism in cases of respiratory diseases, as well as the study of associations among them, are both fundamental for the control of respiratory diseases in poultry production, the development of prevention, and the adaptation of measures and legislation regarding health protection for competitive and sanitary egg production. The present study aimed to investigate the occurrence of MS, MG, OR, AP, PM and IBV, single or in association in laying hens with clinical respiratory signs

MATERIAL AND METHODS

Sample collection

The procedures were approved by the Committee for Ethical Animal Use (CEUA – protocol number 1004) of the "Universidade Federal Fluminense".

Tracheal swabs and blood samples from 14 laying hens' flocks were obtained in the rearing phase and the production phase (10 chickens/flock) at six poultry farms (A-F) from São Paulo, Brazil, totaling 140 tracheal swabs and 140 blood samples (Table 1). The samples were packed in tubes containing phosphate-buffered saline (PBS) with 50% glycerin at the time of collection and kept frozen until processing. All the laying hens presented clinical manifestations such as sneezing, coughing, rales, rhinorrhea, and conjunctivitis. The laying hens were vaccinated against Newcastle disease (at days 7, 35, 70 – live vaccine, HB1 and La Sota strains, drinking water route and 105- inactivated vaccine), Gumboro disease (at days 7, 14, 21 and 35-live vaccine, strain Moulthrop G603, drinking water route), Infectious Coryza (at days 40 and 105 – inactivated vaccine, intramuscular route), avian metapneumovirus (at days 1 and 50- live vaccine, strain 119/95-BR, spray route), Avian Infectious Bronchitis (at days 7, 35, 70 – live vaccines, strain H120, drinking water route, and 105- inactivated vaccine, intramuscular route), *Mycoplasma gallisepticum* (at day 60 – MG-70 strain, spray route).

Table 1 – Identification of farms, production phases, number of flocks sampled, age in weeks and number of samples obtained from laying hens in São Paulo, Brazil.

Farm	Phase	Number of flocks	Age (weeks)	Tracheal swabs	Blood samples
A	Rearing	1	20	20	20
	Production	1	28		
B	Rearing	1	16	20	20
	Production	1	35		
C	Rearing	1	22	20	20
	Production	1	31		
D	Rearing	1	16	20	20
	Production	1	24		
E	Rearing	1	22	20	20
	Production	1	38		
F	Rearing	1	8	40	40
		1	15		
	Production	1	21		
		1	28		
Total				140	140

Molecular detection of pathogens

A 500µl aliquot of the collected sample was submitted to DNA extraction by the phenol-chloroform method (Sambrook and Russel, 2006). Each sample was homogenized and centrifuged at 13.500rpm at 10°C for 20 minutes. After centrifugation, the supernatant was discarded, and 400µL of Tris Ethylenediaminetetraacetic acid (TE) dextrose, 30µl of 10% sodium dodecyl sulfate (SDS) and 30µl of proteinase K 240µg/µl were added to the pellet. The sample was taken to the thermal block at 50°C for 30 minutes with a subsequent ice bath for 5 minutes. Subsequently, 500µL of phenol were added to the samples, homogenized by inversion for 15 minutes, and then centrifuged at 13.500rpm at 10°C for 30 minutes. The supernatant was removed and added to a new microtube with the same volume of chloroform, followed by homogenization for 3 minutes and centrifugation under the same previously stated conditions. The supernatant was removed, added to a 1000µL microtube of ethyl alcohol, and precipitated overnight. The precipitated DNA was centrifuged at 13500rpm at 10°C for 20 minutes and the dried pellet

was resuspended in 100µL of TE buffer, quantified in Biodrop Touch® (Biochrom), and stored at -20°C until further analysis. PCR was performed using primer and conditions described previously by Nascimento *et al.* (1991) for MG, Lauerman *et al.* (1993) for MS, Chen *et al.* (1996) and Chen *et al.* (1998) for AP, Townsend *et al.* (1998) for PM, and Chansiripornchai *et al.* (2006) for ORT. For IBV detection, Access Quick RT-PCR kit (Promega, Madison, WI) were used according to Callison *et al.*, 2006. The reference strains MG ATCC 19610 and MS ATCC 25204, an isolate of OR, and vaccine strains of IBV, AP, PM (Laboratório Biovet S/A, Vargem Grande Paulista, SP) were used as *positive controls*. Reactions were carried out in a thermocycler (Thermo PX-2). The amplicons obtained in PCR were mixed with Gel Red® and submitted to electrophoresis in a 1.5% agarose gel at 94V for 40 minutes, being visualized afterwards using an ultraviolet camera (Nova Instruments).

Serology

ELISA was performed using *M. gallisepticum* and *M. synoviae* Antibody Test Kit (IDEXX, SP, Brazil) according to the manufacturer's instructions.

Statistical analyses

The G-independence test was performed in Bioestat 5.0 to compare the frequency of infection in the rearing and production phases.

RESULTS

A higher frequency of respiratory pathogens was observed by PCR for hens from the production phase compared to those from the rearing phase ($p<0.05$). The frequency of birds infected with MS was higher when compared to the other agents in both phases.

At farm A, 75% of the hens were positive for MS, 10% for MG, 5% (1/20) for IBV, 15% for IBV, and 5% for AP. Only MS was detected at farm B, with a detection rate of 95%. At farm C, 5% were positive for MG and 100% for MS. Farm D presented 35% positivity for MG and 80% for MS, and in farm E 65% were positive for MG and 55% for MS. At farms B, C, D, and E, OR, AP, PM, and IBV were not detected (Table 2). Contrariwise the farm F, 37,5% of the hens were positive for MS, 35% for IBV, and 10% for OR. Furthermore, all samples at this farm were negative for MG, AP, and PM.

Table 2 – Infection frequency as detected by PCR for *Mycoplasma gallisepticum* (MG), *M. synovie* (MS), *Ornithobacterium rhinotracheale* (OR), *Avibacterium paragallinarum* (AP), *Pasteurella multocida* (PM), and by RT-PCR for the Avian infectious bronchitis virus (IBV) in hens from rearing and production phases.

	Rearing phase							Production phase						
Pathogens	Farm A	Farm B	Farm C	Farm D	Farm E	Farm F	Total	Farm A	Farm B	Farm C	Farm D	Farm E	Farm F	Total
MG[1]	1/10 (10%)	0/10 (0%)	1/10 (10%)	1/10 (10%)	5/10 (50%)	0/20 (0%)	8/70 (11,4%)	1/10 (10%)	0/10 (0%)	0/10 (0%)	6/10 (60%)	8/10 (80%)	0/20 (0%)	25/70 (35,7%)
MS[2]	6/10 (60%)	9/10 (90%)	10/10 (100%)	8/10 (80%)	4/10 (40%)	2/20 (10%)	39/70 (55,7%)	09/10 (90%)	10/10 (100%)	0/10 (0%)	8/10 (80%)	7/10 (70%)	13/20 (65%)	47/70 (67,1%)
OR[3]	1/10 (10%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	3/20 (15%)	4/70 (5,7%)	2/10 (20%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/20 (5%)	3/70 (4,3%)
AP[4]	1/10 (10%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/20 (0%)	1/70 (1,4%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/20 (0%)	0/70 (0%)
PM[5]	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/20 (0%)	0/70 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/20 (0%)	0/70 (0%)
IBV[6]	1/10 (10%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	13/20 (65%)	14/70 (20%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/20 (0%)	1/70 (1,4%)

Primer and conditions previously described by: 1-Nascimento *et al.* (1991); 2- Lauerman *et al.* (1993);3- Chansiripornchai *et al.* (2006); 4- Chen *et al.* (1996) and Chen *et al.* (1998); 5- Townsend *et al.* (1998); 6-Callison *et al.*, 2006.

The simultaneous detection of pathogens occurred in 25% of the hens from farm A, 5% of which being of MG and IBV, 5% MG and OR, 5% MS and AP and 10% of MS and OR. At farms C, D, and E, only MG and MS were detected. IBV was detected with MS or OR in 5% of the hens at farm F.

The seroprevalence of MG and MS antibodies was 39.3% (55/140) and 86.4% (121/140) respectively, and no difference was observed in the positivity frequency between the two phases of poultry farming or the prevalence of pathogens (Table 3).

DISCUSSION

Previous reports showed a high prevalence of MS in laying hens in Brazil (Buim *et al.*, 2009; Teixeira *et al.*, 2015) and unlike vaccination for MG, which is widely performed in commercial flocks of laying hens,

Table 3 – Serology results from flocks tested for *Mycoplasma gallisepticum* and *M. synoviae*.

	FARM	MG	MS
REARING	A	10/10 (100%)	10/10 (100%)
	B	10/10 (100%)	10/10 (100%)
	C	9/10 (90%)	2/10 (20%)
	D	10/10 (100%)	10/10 (100%)
	E	8/10 (80%)	10/10 (100%)
	F	11/20 (55%)	12/20 (60%)
PRODUCTION	A	10/10 (100%)	10/10 (100%)
	B	10/10 (100%)	10/10 (100%)
	C	6/10 (60%)	9/10 (90%)
	D	10/10 (100%)	10/10 (100%)
	E	10/10 (100%)	10/10 (100%)
	F	9/20 (45%)	18/20 (90%)
TOTAL		113/140 (80.7%)	121/140 (86.4%)

vaccination for MS is not commonly performed in Brazil. The subclinical course of many MS infections can lead to the continued presence of this microorganism in the farm (Nascimento *et al.*, 2005), a relevant issue since mycoplasmas, especially MS, are able to cause immunosuppression (Stipkovits & Kempf 1996), as well as economic losses. In this work, all layer hens presented respiratory signs and all farms were MS positive by PCR. Although MG was detected by PCR in farms A, C and E, this can be explained by the vaccination protocols, since live attenuated vaccines are widely used for immunization against this pathogen. This hypothesis can be supported by the high detection of antibodies against MG even in flocks negative by PCR. The characterization of these strains with genomic methods or specific primers would be necessary to confirm them as either field or vaccine strains.

IBV was only detected in rearing flocks from farms A and F. Live-attenuated vaccines against IBV were used in rearing chickens and this type of vaccine can result in replication in the respiratory epithelium tissue, making a RT-PCR detection possible. This can explain the high IBV detection rate in farm F's rearing flocks, since the samples were taken at the 8th and 15th weeks of rearing and the vaccination with live vaccines for IBV occurs at the 10th and 15th. Differentiation between vaccine and field strains was not performed in this study but could be done using specific primers or selected gene sequencing and phylogenetic evaluation. Synergism between the IBV vaccine strain and MS or other pathogens is known (Kleven *et al,* 1972; Pang *et al.*, 2002; Matthijs *et al.*, 2009; Sid *et al.*, 2015) and may exacerbate respiratory signs, resulting in lower poultry performance. Another significant factor is that even with extensive vaccination, avian infectious bronchitis outbreaks in commercial poultry remain a significant problem. New coronavirus serotypes and variants emerge continuously, forcing poultry producers and animal health pharmaceutical companies to constantly evaluate their vaccination plans and develop new vaccines (Jordan, 2017).

The distance between the studied farms varied between 600m and 6km, so the high density of laying hens in the study area can facilitate the spread of pathogens among farms, demanding that they maintain a strict biosecurity program to prevent the entry of pathogens. The study in the same region by Correzola *et al.* (2012) also reported a high seroprevalence of MG and highlighted the density and proximity of poultry farms. Moreover, this study reported that mycoplasmosis control in this region is performed mainly through vaccination and not through biosecurity measures. Batista *et al.* (2020) detected a high seroprevalence for MG, MS, and IBV in backyard chickens raised near commercial farms in the state of Minas Gerais. The presence of these pathogens reinforces that vaccinations protocols should be performed with biosecurity measures to prevent the entry and spread of pathogens in the poultry farms. It is also important to consider that pathogens and vaccine strains will also spill-over to family poultry and spill-back to the industrial poultry, as a two-way relationship.

The effect of mycoplasma species and others respiratory pathogens or vaccine strains may lead to a higher mortality rate, uneven flocks, costs with antibiotics, drop in egg production, and condemnation of carcasses. In Argelia, Sid *et al.* (2015) also detected MG, MS, and IBV in a commercial flock with an increase in mortality rate and respiratory signs, but unlike our study, a higher frequency of MG as compared to MS was identified, possibly due to the absence of MG vaccination in that country. However, when analyzing 117 oropharyngeal swabs from different bird species in Ethiopia, Hutton *et al.* (2017) detected high positivity rates for MS, MG, and IBV, and MS was the most prevalent, as in this study.

It was also possible to detect OR and AP by PCR in some flocks. The occurrence of several etiological agents that cause respiratory diseases in laying hens was confirmed by PCR and serology, with MS being the most prevalent, present in all farms studied.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

ABPA – Associação Brasileira de Proteína Animal. Relatório anual 2020. São Paulo; 2020. Disponível em: http://abpa-br.org/wp-content/uploads/2020/05/abpa_relatorio_anual_2020_portugues_web.pdf

Batista IA, Hoepers PG, Silva MFB, Nunes PLF, Diniz DCA, Freitas AG, *et al.* Circulation of major respiratory pathogens in backyard poultry and their association with clinical disease and biosecurity. Brazilian Journal of Poultry Science 2020;22(1):1-12.

Buim MR, Mettifogo E, Timenetsky J, Kleven S, Ferreira AJP. Epidemiological survey on *Mycoplasma gallisepticum* and *M. synoviae* by multiplex PCR in commercial poultry. Pesquisa Veterinária Brasileira 2009;29(7):552–556.

Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, *et al.* Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. Journal of Virology Methods 2006;138(1-2):60-65.

Chansiripornchai N, Wanasawaeng W, Sasipreeyajan J. Seroprevalence and identification of *Ornithobacterium rhinotracheale* from broiler and broiler breeder flocks in Thailand. Avian Diseases 2007;51(3):777–780.

Chen X, Chen Q, Zhang P, Feng W, Blackall PJ. Evaluation of a PCR test for the detection of *Haemophilus paragallinarum* in China. Avian Pathology 1998;27(3):296–300.

Chen X, Miflin JK, Zhang P, Blackall PJ. Development and application of DNA probes and PCR tests for *Haemophilu sparagallinarum*. Avian Diseases 1996;40(2):398–407.

Correzola LM, Buchala FG, Vitagliano SMM, Jordão RS, Buim MR, Fava CD. Reações sorológicas contra *Mycoplasma gallisepticum* em aves de postura de granjas comerciais no Estado de São Paulo. Ars Veterinaria2012;28:41-47.

Hutton S, Bettridge J, Christley R, Habte T, Ganapathy K. Detection of infectious bronchitis virus 793B, avian metapneumovirus, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in poultry in Ethiopia. Tropical Animal Health and Production 2017;49(2):317-322.

Jordan B. Vaccination against infectious bronchitis virus: A continuous challenge. Veterinary Microbiology 2017;206:137-143.

Kleven SH, King DD, Anderson DP. Airsacculitis in broilers from *Mycoplasma synoviae*: effect on air-sac lesions of vaccinating with infectious bronchitis and Newcastle virus. Avian Diseases 1972;16:915–924.

Lauerman LH, Hoerr FJ, Sharpton AR, Shah SM, van Santen VL. Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. Avian Diseases 1993;37(3):829-834.

Matthijs MGR, Ariaans MP, Dwars RM, van Eck JHH, Bouma A, Stegeman A, *et al.* Course of infection and immune responses in the respiratory tract of IBV infected broilers after superinfection with *E. coli*. Veterinary Immunology and Immunopathology 2009;127(1–2):77–84.

Nascimento ER, Pereira VLA, Nascimento MGF, Barreto ML. Avian mycoplasmosis update. Brazilian Journal of Poultry Science 2005;7(1):1–9.

Nascimento ER, Yamamoto R, Herrick KR, Tait RC. Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. Avian Diseases 1991;35(1):62-69.

Pang Y, Wang H, Girshick T, Xie Z, Khan MI. Development and application of a multiplex polymerase chain reaction for avian respiratory agents. Avian Diseases 2002;46(3):691-699.

Sambrook J, Russell DW. Purification of nucleic acids by extraction with phenol: chloroform. CSH Protocol 2006;2006(1):pdb.prot4455.

Sid H, Benachour K, Rautenschlein S. Co-infection with multiple respiratory pathogens contributes to increased mortality rates in Algerian poultry flocks. Avian Diseases 2015;59(3):440-446.

Stipkovits L, Kempf I. Mycoplasmoses in poultry. Revue Scientifique et Technique (International Office of Epizootics) 1996;15:1495-1525.

Teixeira VCM, Baptista D de Q, Carlos FC, Menezes WR de, José DS, Barreto ML, *et al.* Situação epidemiológica da micoplasmose aviária no Estado do Rio de Janeiro. Brazilian Journal of Veterinary Medicine 2015;37(4):379-385.

Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJ. Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. Journal of Clinical Microbiology 1998;36(4):1096-1010.