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Epidemiological survey on *Mycoplasma gallisepticum* and *M. synoviae* by multiplex PCR in commercial poultry[1]

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ABSTRACT.- Buim M.R., Mettifogo E., Timenetsky J., Kleven S. & Ferreira A.J.P. 2009. **Epidemiological survey on *Mycoplasma gallisepticum* and *M. synoviae* by multiplex PCR in commercial poultry.** *Pesquisa Veterinária Brasileira 29(7):552-556.* Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP 05508-900, Brazil. E-mail: ajpferr@usp.br

Mycoplasmas are important avian pathogens, which cause respiratory and joint diseases that result in large economic losses in Brazilian and world-wide poultry industry. This investigation regarding the main species of mycoplasmas, *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS), responsible for the above mentioned conditions, was carried out through PCR Multiplex analysis. One thousand and forty-six (1,046) samples of tracheal swabs and piped embryos were collected from 33 farms with laying hens, breeders, broilers or hatchery, located in the Brazilian states of São Paulo, Paraná and Pernambuco, where respiratory problems or drops in egg production had occurred. The MG and MS prevalence on the farms was 72.7%. These results indicated (1) high dissemination of mycoplasmas in the evaluated farms, with predominance of MS, either as single infectious agent or associated with other mycoplasmas in 20 farms (60.6%), and (2) an increase of MS and decrease of MG infection in Brazilian commercial poultry.

INDEX TERMS: *Mycoplasma gallisepticum*, *M. synoviae*, PCR, epidemiology, chicken.

RESUMO.- [**Investigação epidemiológica de *Mycoplasma gallisepticum* e *M. synoviae* por PCR Multiplex em estabelecimentos comerciais de aves.**] Os Micoplasmas são importantes patógenos aviários que causam doenças respiratórias e de articulações que resultam em grandes perdas econômicas para a indústria avícola brasileira e mundial. O estudo das principais espécies de Mycoplasma, *Mycoplasma gallisepticum* (MG) e *M. synoviae* (MS), responsáveis pelas doenças mencionadas acima, foram analisadas pela técnica de PCR Multiplex. Foram colhidas 1046 amostras de suabe traqueal e embriões bicados de 33 estabelecimentos de aves de postura, matrizes, frangos de corte e um incubatório, localizados nos Estados brasileiros de São Paulo, Paraná e Pernambuco, as quais apresentavam problemas respiratórios ou queda na produção de ovos. A prevalência de MS e MG nas granjas foi de 72,7%. Os resultados indicaram uma alta disseminação de *Mycoplasma* nas granjas avaliadas, com predominância de MS, como um único agente infeccioso ou associado com outros micoplasmas em 20 granjas (60,6%). Assim, este estudo indicou o aumento da incidência de MS e a redução de MG nas granjas comerciais no Brasil.

TERMOS DE INDEXAÇÃO: *Mycoplasma gallisepticum*, *M. synoviae*, PCR, epidemiologia, galinha.

INTRODUCTION

Poultry industry is an activity that targets the production of animal protein of excellent quality and at low costs, and stands out in national economy for generating jobs and

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foreign exchange credits for the balance of trade. Since 2004, Brazil has taken the first place in the product's world trade, consolidating this sector as the second one in exports in the Brazilian agribusiness ranking. The production and commercialization of eggs, chickens and other birds correspond to about 2.5% of the Brazilian National Product Growth.

Mycoplasmas are important avian pathogens responsible for chronic respiratory diseases (CRD) and infectious synovitis or bursitis of chickens and turkeys, which result in large economic loss for the world poultry industry. Among the 20 species of mycoplasmas isolated from birds, *Mycoplasma gallisepticum* (MG), *M. synoviae* (MS), *M. meleagridis* (MM) and *M. iowae* (MI) are considered the main pathogenic agents for chickens and turkeys. These agents cause economic losses due to decrease in growth rate, loss of weight, condemnations at the slaughterhouse (due to CRD in broilers) and to decrease in egg production (Hoerr et al. 1994). Control programs and vaccination account for additional costs.

The control programs, which may include surveillance (serology, culture, isolation, and identification) for MG and MS, must be performed, manly in breeder flocks. Although recommended by the National Program for Avian Health (NPAH) as a definitive method, mycoplasma isolation is difficult, expensive and time consuming. Techniques for the detection and analysis of DNA through Polymerase Chain Reaction (PCR) arise as a very interesting alternative of diagnosis method, because they offer sensitivity, specificity, capability of accomplishment of exams on a large scale (Nascimento et al. 1991) and, nowadays, economical viability.

The sensitivity observed in PCR is important for detection of pathogenic agents in clinical samples taken from asymptomatic animals, or those undergoing antibiotics treatment. Furthermore, it is possible to detect a pathogenic agent before the host's immunologic response, or in host with immunity depression, demonstrating advantages also over the serologic tests (Garcia et al. 1995, Kempf 1998).

The objective of this study was to evaluate the prevalence of mycoplasmas in different production system of commercial Brazilian poultry by the Multiplex PCR.

MATERIALS AND METHODS

Mycoplasma strains. *Mycoplasma synoviae* reference strain (MS WVU-1853) was kindly supplied by Dr. Elmiro R. Nascimento, of the Federal Fluminense University, Niterói, RJ. *M. gallisepticum* strains used were the S6, 496-A, 695-I, 5969-A and R (kindly supplied by Dr. S.H. Kleven, from Athens University, Georgia, USA), the Conn-F[6] and the Ts-11[7] as a vaccinal strains. Other species of mycoplasmas commonly isolated from chickens were also utilized to verify possible cross-reactions: *M. gallinarum*, *M. gallinaceum, M. iowae, M. meleagridis* and *A. laidlawii* (supplied by Dr. S.H. Kleven).

Sampling. One thousand and forty-six (1,046) samples were collected from commercial poultry of the laying, breeders and broilers of different age groups, coming from three States of Brazil (São Paulo, Paraná and Pernambuco), from 2001 to 2004. The samples belonged to flocks that showed respiratory and/or decreasing egg-production problems.

Samples collection. Field material was collected by rubbing sterile swabs against the tracheal wall for removing of the mucosa cells. These materials were transported into Frey medium (Frey et al. 1968), specific for the isolation of mycoplasmas. From necropsied hens or chickens, samples were collected from tracheae, lungs, thoracic and abdominal air sacs.

DNA extraction. Mycoplasmas strains and isolates were submitted to the DNA extraction methods described by Fan et al. (1995). Accordingly, this method was employed in all collected samples. Swabs or organ fragments were submerged into 5ml of Frey liquid medium and incubated over-night at 37ºC. Afterwards, 1ml of the cultures was centrifuged at 10,000 x g for 10 minutes at 4ºC; the sediment was washed twice in 100µl of saline buffered at 150mM Phosphate Buffered Saline (PBS) pH 7.2 and homogenized into 25µl of the same buffer. Following, cell suspension was heated up to 100ºC for 10min and ice-cooled for 5min. Finally, it was centrifuged again for 6 minutes, and the supernatant containing DNA was stocked at 4ºC.

Standardization of Multiplex PCR. The method used was the one described by Mettifogo et al. (2001). Multiplex PCR was applied for simultaneous detection of the mycoplasmas species most important for poultry industry - *M. gallisepticum* and *M. synoviae* - besides the differentiation of the Conn-F *M. gallisepticum* strain. The PCRs were accomplished following precaution procedures according to Kwok & Higuchi (1989). Primers used on the multiplex PCR reactions are listed in Table 1.

Table 1. Primers used in the Multiplex PCR reactions with their respective sequences, amplified product size and reference

Primers[a]	Sequence	Product	Reference
MG-f	GGATCCCATCTCGACCACGAGAAAA	732 pb	Nascimento
MG-r	CTTTCAATCAGTGAGTAACTGATGA		et al. (1991)
MGF-f	TAACCCTTCATCACCTCATCTAGAG	524 pb	Nascimento
MGF-r	CTGTTTGCTAAAGAACAAGTTGATC		et al. (1993)
MS-f	GAGAAGCAAAATAGTGATATCA	207 pb	Lauerman
MS-r	CAGTCGTCTCCGAAGTTAACAA		et al. (1998)

[a] f = *forward*, r = *reverse.*

PCR cycle. The first extension stage was at 94ºC for 5min and 35 cycles at 94ºC for 1min, at 55ºC for 1min and at 72ºC for 2min. Final extension stage was at 72ºC, for 10min. After amplification, products were subjected to 60min electrophoresis in 1.0% agarose gel containing 0.5µg/mL ethidium bromide, under 80-Volt. Electrophoresis gels were carried out in 1x TAE (EDTA Tris acetic acid) buffer, exposured to ultra-violet light, and photographed by photo documentation[8].

Statistical analysis. The Q^2 test was performed on the laying and breeder flocks on confidence interval of the p=5%. These flocks were divided into three age groups: (a) 1-25, (b) 25-50, and (c) 50-75 weeks old.

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RESULTS

The Multiplex PCR test applied on 1,046 samples from 33 commercial poultry demonstrated a higher presence of the major avian mycoplasmas strains, *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) (Tables 2-4). Table 2 shows the results obtained from the collected samples, without considering type of farm or flock, and evidences 38.91% of mycoplasmas. The number of flocks (n=70) positive for mycoplasma was 44 (62.85%), not considered the type of production system or the farm to which they belonged (Table 3). On the other hand, 24 farms (72.7%) were positive for one of the species of mycoplasma, being higher in the layers (Table 4). The occurrence of MS prevailed over MG in all flocks or farms. Statistical analysis showed no significant difference between age groups, regarding the occurrence of MS and MG in the layer or breeding flocks.

Table 2. Multiplex PCR analysis of mycoplasma prevalence in samples from birds in different commercial poultry systems from 2001 to 2004

Production system	MS (%)	MG (%)	MG-F (%)	Associations[a] (%)	Negative (%)	Samples positive for any mycoplasma (%)	Total
Layers	208 (19.88)	2 (0.19)	11 (1.05)	10 (0.95)	180 (17.2)	231 (22.08)	411
Breeders	130 (12.46)	14 (1.33)	19 (1.81)	1 (0.09)	336 (32.12)	164 (15.67)	500
Broilers	1 (0.09)	5 (0.47)	0	0	123 (11.75)	6 (0.57)	129
Hatchery	0	0	6 (0.57)	0	0	6 (0.57)	6
Total	339 (32.4)	21 (2.0)	36 (3.44)	11 (1.05)	639 (61.08)	407 (38.91)	1046

[a] Associations: MG + MG-F + MS.

Table 3. Multiplex PCR analysis of mycoplasma prevalence in commercial poultry flocks from 2001 to 2004

Production system	MS (%)	MG (%)	MG-F (%)	Associations[a] (%)	Negatives (%)	Flocks positive for any mycoplasma (%)	Total of flocks
Layers	16 (22.85)	0	0	7 (10.0)	6 (8.57)	23 (32.85)	29
Breeders	14 (20.0)	2 (2.85)	0	2 (2.85)	15 (21.42)	18 (25.71)	33
Broilers	1 (1.42)	1 (1.42)	0	0	5 (7.14)	2 (28.57)	7
Hatchery	0	0	1 (1.42)	0	0	1 (1.42)	1
Total	31 (44.28)	3(4.28) (1.42)	1 (12.85)	9 (37.14)	26 (62.85)	44	70

[a] Associations: MG + MG-F + MS.

Table 4. Multiplex PCR analysis of mycoplasma prevalence in farms from 2001 to 2004

Production system	MS (%)	MG (%)	MG-F (%)	Associations[a] (%)	Negatives (%)	Flocks positive for any mycoplasma (%)	Total
Layers	5 (15.15)	0	0	6 (18.18)	3 (9.09)	11 (33.33)	14
Breeders	5 (15.15)	2 (6.06)	0	2 (6.06)	4 (12.12)	9 (27.27)	13
Broilers	1 (3.03)	1 (3.03)	0	1 (3.03)	2 (6.06)	3 (9.09)	5
Hatchery	0	0	1 (3.03)	0	0	1 (3.03)	1
Total	11 (33.33)	3 (9.09)	1 (3.03)	9 (27.27)	9 (27.27)	24 (72.72)	33

[a]Associations: MG + MG-F + MS.

DISCUSSION

The multiplex PCR technique used in this study indicated a high prevalence of mycoplasma in the evaluated farms (72.72%), with *Mycoplasma synoviae* (MS) predominance, mainly in layer hens and breeders with respiratory problems (Table 4). According to evaluation of flocks, without considering the farm to which they belonged, mycoplasma presence was evidenced with high incidence: 62.85% (Table 3). These two evaluation modes indicated that mycoplasmas are widely distributed among farms and flocks, especially among layers and breeders.

According to the number of collected samples, regardless of flocks or farms to which they belonged, the occurrence was of 38.91% (Table 2). This index represents the pathogen's horizontal transmissibility characteristics among birds of a same flock. Although high, it was lower than mycoplasma occurrence in the analyses accomplished considering flocks and farms. Consequently, mycoplasmosis does not display high horizontal transmissibility, when compared with diseases as Influenza or Newcastle, which may infect even up to 100% of birds of a same flock within a few days.

Considering the already acknowledged vertical transmission characteristics of this disease (Mohammed et al. 1987), the prevalence of 27% of the mycoplasmas in the breeder farms (Table 4) can imply in continuous dissemination for the commercial farms that receive chicks, what amplifies the disease occurrence and emphasizes their effects regarding economical losses.

In relation to the presence of mycoplasmas in layers farms, their concentration in some regions and inexistence of adequate sanitary barriers that may enable the isolation of farms are predisposing factors for the disease dissemination. Other contributing factors are related to the resistance to antimicrobial treatments, and to the immunologic system escape mechanisms that these pathogens make use of. The mycoplasmas can remain feasible for long periods, making possible infection of new flocks introduced into the farms. Layers and breeders remain on

the farm for long periods, during which they go through different production stages. These long periods render them susceptible to several pathogenic agents that interfere on the birds' defense system and predispose them to occurrence of infection. One of the main characteristics of mycoplasmosis, especially the one caused by MS, is an asymptomatic manifestation, although it induces damages to the infected host's health, and may even cause suppression of their immune system (Kleven et al. 1991, Stipkovits & Kempf 1996).

Regarding the MS and considering age group, there was no significant statistical difference in occurrence in layer birds and breeders. Consequently, birds may get infected in any of the production stages.

As for *Mycoplasma gallisepticum* (MG), the relatively low occurrence in layer birds is probably due to the intense control of this agent, which has been conducted for several years already, especially through vaccination. In Brazil, reduction of this kind of mycoplasmas in our stocks began in the 1980s and, additionally, it was boosted by the NPAH created in 1984, whose precautions certainly have collaborated to decrease the disease indices.

The high MS occurrence in layer birds probably is due to the fact that the vaccine against is still very little used, and none of the farms studied made use of it. Some papers with MS have been published in Brazil, such as of economical impacts (Balem & Fiorentin 1990), pathogenicity determination of autochthonous MS strains in chicken embryos (Fiorentin & Jeanisch 1994), MS diagnosis establishment by PCR (Silveira et al. 1996), and MS and MG eradication from flocks of breeder hens with antibiotic treatment of eggs, in combination with other control measures (Fiorentin & Jeanisch 1994, Nascimento & Nascimento 1994).

The MG-F strain was detected at a layer farm, at the hatchery and in two breeder farms. In the first case the laying hens that had been vaccinated with this strain did not show any clinical symptom and were in good production levels. This capability of distinguishing the F-strain from other strains, through PCR, makes possible the application of this method in monitoring programs, as well as in evaluations of the vaccine performance in laying hen farms. In the second case, the MG-F strain was isolated and detected by the PCR in embryos in a hatchery, with decrease of hatchability indices, due to embryonic mortality. These embryos exhibited arthritis and, in fact, one of the MG-F isolates was obtained from the synovial fluid with these characteristics. The MG-F strain shows moderate virulence for adult birds, but in young fowls it does display pathogenicity, causing the already described classical mycoplasmosis respiratory and joint symptoms (Kleven & McMartin 1980). In the third case, the MG-F strain was detected in two breeder farms that did not use vaccination programs against mycoplasmas. These birds did not display respiratory symptoms or decreased egg production indices, but evidenced serologic reactions in the RSA and ELISA tests for MG, previously performed in other laboratories.

Mycoplasmosis prevalence has been reported in Brazil for some years, whether in isolated form - in certain states - or from data gathered in broader studies. In general, one may observe that MS prevalence has been superposing that of MG throughout the years. In the 1960s and 1970s, in the State of Minas Gerais, 25.7% and 32.8% MG prevalence were found, obtained through isolation and serology, respectively (Resende et al. 1968, Reis et al. 1973). In the State of Goiás, study of condemnation cases in broiler hens due to airsacculitis, focusing on main causative agents, detected 32% of MG, 25% of MS, besides 16% of *Escherichia coli* and MS associations (Minharro et al. 2001). In a paper on serologic monitoring with wide sampling of breeder chickens from various states in Brazil, from 2003 to 2004, Cardoso et al. (2006) demonstrated 1.58% MS prevalence, and absence of MG.

Bearing in mind that breeders or breeder flocks must be free of infections caused by pathogenic mycoplasmas, the 27.27% occurrence found in the farms studied herein is quite worrying. For the breeder farms that participate in the NPAH, vaccinations are not allowed, and there exists a strict control regarding sanity and biosafety. Treatment with antibiotics does not eliminate mycoplasma infections and is not the suitable measure for this agent control. Consequently, in the case of occurrence in breeder flocks, the recommendation for eradication of this disease is the elimination of infected flocks. In cases of farms that do not take part in the NPAH, theses requirements are not necessarily fulfilled and, for this reason, infections perpetuate on and on in the production chain.

In this study we used the Multiplex PCR method, which simultaneously detects the two main mycoplasma pathogenic species (MS and MG) besides differentiating the MG-F vaccinal strain. The specificity of PCR presents to be superior to serologic tests and isolation. As to economical viability, PCR costs have been decreasing and can already be compared to those of HI and ELISA. Cost-benefit relation of this method has already made it attractive enough to be implanted in routine diagnostic laboratories, thus being considered a definitive method.

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