

## Transmission, serologic and tissue responses in chickens vaccinated with *Mycoplasma gallisepticum* F strain (MG-F)<sup>1</sup>

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MG-F protects chickens from MG Mycoplasmosis and monitoring is done by serology (SAR and ELISA) and PCR. Histopathology is used to evaluate bird response to MG. This study evaluated MG-F profile vaccination in SPF chicken. This trial used 100 chickens, being 40 unvaccinated (G1), 40 eye-drop vaccinated at 8 weeks of age with MG-F (Ceva Animal Health, São Paulo, SP, Brazil) (G2) and 20 immunized by contact (G3). Samples were obtained on the 8th, 12th, 15th, 18th, 20th and 24th week for SAR, ELISA and PCR. Fragments of trachea and air sac, for microscopy, were got after necropsies on the 15th and 24th week. Up to 12 weeks there was no significant difference among groups by SAR. SAR reactions appeared from the 15th week with these averages: G1 (1.7, 1.76, 0.1, 0.15), G2 (7.81, 7.65, 8.25, 6.29) and G3 (8.1, 8.5, 9.5, 6.16), while by ELISA it occurred after the 18<sup>th</sup> week with optical densities averages: G1 (0.19, 0.14, 0.16), G2 (0.47, 0.45, 0.41) and G3 (0.55, 0.51, 0.51). Positivity in G3 by PCR occurred seven weeks after exposure. At the 15th week the air sac score means were 0.20, 0.55, and 0.32 and 24th week were 0.15, 0.80 and 0.66 ( $p>0.05$ ). For trachea, G2 (0.48) yielded higher score average than G1 (0.10) and G3 (0.00) on the 15th week. Changes in G3 were seen only at 24th week, being this average (1.00) significantly different ( $p<0,05$ ) from G1 (0.08) and G2 (0.46). SAR and PCR detected MG-F in G3 earlier than ELISA. Higher tracheal changes for G2 and G3 as compared to G1 could be ascribed to MG-F vaccine infection.

INDEX TERMS: *Mycoplasma gallisepticum* F strain, MG-F, transmissibility, serology, PCR, histopathology.

**RESUMO.- [Transmissão, resposta sorológica e tecidual de poedeiras vacinadas com *Mycoplasma gallisepticum* cepa F.]** *Mycoplasma gallisepticum* cepa F (MG-F) é altamente utilizada em vacinação de poedeiras. MG-F confere bons níveis de proteção às galinhas, deslocando MG de campo ou diminuindo o número deles no trato respiratório. Soroaglu-

tação Rápida (SAR), ELISA e PCR são testes no monitoramento da micoplasmose, enquanto a histopatologia, mesmo não sendo rotineira, é usada para avaliar a resposta das aves à infecção por MG. O objetivo deste estudo foi avaliar a transmissibilidade, soroconversão e alterações teciduais de MG-F em galinhas. Um total de 100 galinhas SPF foi utilizado, sendo 40 delas não vacinadas (G1), 40 vacinadas na 8<sup>a</sup> semana de idade com MG-F (Ceva Saúde Animal, São Paulo/SP, Brasil) (G2) e 20 imunizadas por contato com aves do G2 (G3). Soros e suabes traqueais foram obtidos na 8<sup>a</sup>, 12<sup>a</sup>, 15<sup>a</sup>, 18<sup>a</sup>, 20<sup>a</sup>, 24<sup>a</sup> semana para monitoramento por SAR, ELISA e PCR. Fragmentos de traqueia e saco aéreo, para microscopia, foram feitas após necropsias na 15<sup>a</sup> e 24<sup>a</sup> semana. Até a 12<sup>a</sup> semana não houve diferença significativa entre os grupos pela SAR. Houve reação a SAR a partir da 15<sup>a</sup> semana com as seguintes médias: G1 (1,7; 1,76; 0,1; 0,15), G2

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(7,81; 7,65; 8,25; 6,29) e G3 (8,1; 8,5; 9,5; 6,16), enquanto por ELISA a soroconversão ocorreu a partir da 18ª semana com médias de densidades óticas de G1 (0,19; 0,14; 0,16), G2 (0,47; 0,45; 0,41) e G3 (0,55; 0,51; 0,51). Todas as aves do G3 apresentaram positividade pela PCR sete semanas após exposição. Não houve diferença significativa entre as médias dos escores de saco aéreo entre os grupos, na 15ª semana (0,20; 0,55; 0,32) e 24ª semana (0,15; 0,80 e 0,66). Em relação à traqueia, G2 apresentou média maior na 15ª semana (0,48) que G3 (0,00) e G1 (0,10). Alterações em G3 foram observadas somente na 24ª semana onde as médias foram de 0,08(G1); 0,46 (G2) e 1,00 (G3), havendo significância ( $p < 0,05$ ) entre G1 e G3. SAR e PCR foram capazes de detectar a transmissão de MG-F de forma precoce em relação ao ELISA. Em relação ao G1 (controle negativo) as reações teciduais para os grupos vacinados foram mais intensas na 24ª semana, o que tudo indica sendo resposta à vacinação.

TERMOS DE INDEXAÇÃO: *Mycoplasma gallisepticum* cepa F, MG-F, transmissão, sorologia, PCR, histopatologia.

## INTRODUCTION

*Mycoplasma gallisepticum* (MG) is a highly infectious respiratory pathogen that affects poultry. MG infection causes respiratory rales, nasal discharge, coughing, and occasionally conjunctivitis in chickens. The most prominent pathological findings include inflammatory lesions in trachea, air sacs, lungs, conjunctiva, and other tissues such as the oviduct (Charlton et al. 1996, Levisohn et al. 2000). Significant economic losses from MG infection in poultry occur due to reduced egg production and hatchability, as well as downgrading of carcasses (Charlton et al. 1996). MG transmission can occur both horizontally through aerosols and vertically through the egg, leading to a rapid spread within the flock (Nascimento & Pereira 2009).

Serological examinations, culturing, polymerase chain reaction (PCR) and immunohistochemical methods are used in the diagnosis of mycoplasma infections (Nascimento & Pereira 2009). In spite of not using frequently, gross and microscopic examinations have been utilized to help the diagnosis of avian mycoplasmosis in naturally infected birds and are similar to the lesions described in experimentally infected birds (Yoder Jr 1991, Ley & Yoder 1997).

Control of pathogenic avian mycoplasmas can consist of one of three general approaches: Maintaining flocks free of infection, medication, or vaccination. Maintaining flocks free of pathogenic mycoplasmas consists of getting replacements from mycoplasma-free sources in a single-age, all-in all-out management system. Good biosecurity and an effective monitoring system are necessary aspects of this program. Medication can be very useful in preventing clinical signs and lesions, as well as economic losses, but cannot be used to eliminate infection from a flock and is therefore not a satisfactory long-term solution. Vaccination against *Mycoplasma gallisepticum* (MG) can be a useful long-term solution in situations where maintaining flocks free of infection is not feasible, especially on multi-age commercial sites. Previously, bacterins and live vaccines have been used commercially for the control of *M. gallisepticum* infection in

chickens. Killed whole-cell *M. gallisepticum* bacterins reduced the severity of lesions and egg production losses but did not completely prevent *M. gallisepticum* colonization of the chicken respiratory tract upon challenge (Kleven 1986, Yagihashi 1986). At the same time, bacterins fail to provide complete protection against heterologous exposure. Over the past decade, the use of bacterins has been supplanted by more effective live attenuated vaccines.

Attenuated vaccines stimulate immune responses by cellular and humoral basis and act as an instrument of competitive exclusion in relation to field MG strains (Cummings & Kleven 1986, Whithear 1996). There are four live MG vaccine types available, i.e., F-Conn strain (MG-F), ts-11, 6/85 and MG-70 which can reduce drop in egg production, although they are not able to prevent transovarial transmission (Carpenter et al. 1981, Glisson & Kleven 1984). The ts-11 and 6/85 strains induced a milder post-vaccination reaction than F-strain, produced a weaker serological response, and did not persist as long in the upper respiratory tract. The F-strain-vaccinated chickens had the fewest and mildest air-sac lesions post-challenge, the 6/85 and ts-11 groups had somewhat less protection against airsacculitis (Abd-El-Motelib & Kleven 1993).

The aim of this study was to evaluate MG-F profile vaccination (transmission to contact birds, serologic and tissue responses in trachea and air sac of SPF chicken).

## MATERIALS AND METHODS

**Pullet housing and management.** This experiment was conducted in premises located in Cachoeiras of Macacu-RJ, Brazil, starting with 100 1-day-old chicks from SPF eggs hatched on site. During the experiment, the birds were housed in isolated experimental room units. The rooms measured 3x3 m, with roof clay and millimeter screens on window and door and *Polyvinyl chloride* (PVC) lining under the roof. Feed and water were provided ad libitum throughout the experimental period by the use of tubular feeders and bell-shaped drinkers. The feed formulation followed the nutritional requirements of each stage of rearing, with natural day light. The experiment was authorized by the permission number 155 from the Animal Care Ethic Committee of the Fluminense Federal University, Niterói/RJ, Brazil.

**Experimental design.** A total of 100 leghorn chickens were used, being 40 unvaccinated (Group 1), 40 eye-drop vaccinated at 8 weeks of age with MG - F (Ceva Animal Health, São Paulo, SP, Brazil) (Group 2) and 20 immunized by contact (Group 3).

**Serology.** Blood samples for serum obtention were collected at 8, 12, 15, 20, 24, 27, 30 and 33 weeks of age for SAR and Elisa serology. Approximately 3.0 mL of blood were collected from the brachial vein of each bird. The sera obtained were immediately tested for SAR against MG antigens according to the manufacturer's instructions (Intervet, SP, Brazil). Undiluted positive sera by SAR were considered suspicious and were therefore diluted further and retested. Serum was considered positive if the positivity was seen at a dilution of 1:10, according to the guidelines of the National Poultry Health (National Poultry Health Program, PNSA, Brazil, 1994). Sera received the following scores: negative gross serum (0), diluted serum positive (1) positive serum dilution 1:5 (5) and positive serum at 1:10 dilution (10). The variation of the intensity of the reaction was investigated by analysis of variance (ANOVA).

MG ELISA was analysed by *M. gallisepticum* Antibody Test Kit (IDEXX, SP, Brazil). The results obtained were considered positive when the optical density (OD) was equal to or greater than 0.2.

**Necropsy.** Chickens were euthanized and necropsied at the 15th and 24th week of age by cervical dislocation according to the resolution number 1000 of the Veterinary Medicine Federal Council (CFMV). Samples from tracheas and air sacs were analysed from gross examination and fixed in 10% neutral buffered formalin and submitted for histological analysis. These tissues were routinely processed, embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin according to standard histological protocols. Sections of trachea and air sacs were examined microscopically. Tracheal lymphocytic infiltrates and air sac were scored using the system of Nunoya et al. (1987) (24) and Papazisi et al. (2002) with minor modifications (27).

**Airsac lesion score.** Airsac lesion scores were grossly evaluated according to the following criteria: 0: normal air sac, clear and thin; 1 = slightly cloudy and/or dark with slight thickening; 2 = medium turbidity and/or with yellow exudate, often foamy; 3 = severe exudation and thickening; 4 = severe airsacculitis with considerable exudates. By airsac microscopy, the scores were: 0: no changes; 1: discrete nodular lesion with few heterophils; 2: nodular lesion with infiltration of heterophils; 3: diffuse nodular lesions with heterophils.

**Tracheal lesion score.** Trachea were grossly examined and lesions were graded by the following scores: 0: Normal; 1: hiperemic or petequiae, often some mucous; 2: enough mucous present; 3: mucous in excess; 4: mucous in excess and thickening. By tracheal microscopy scoring were: **0:** no lesion; **1:** 1-3 discrete lymphoid aggregates (DLA) without submucosa invasion or at least one of them invading the submucosa, or four or more ALD with or without invasion of the submucosa; **2:** DLA (regardless of the number and the invasion of the submucosa) associated with one or two lymphoid aggregates in follicular pattern (LAFP) without scattering to the lamina propria; **3:** DLA (regardless of the number and the invasion of the submucosa) associated with one or two LAFP with submucosa invasion; and/or discrete confluence among focus and / or follicles; **4:** DLA (regardless of the number and the invasion of the submucosa) associated with three or more LAFP with submucosa invasion with or without invasion of the submucosa and / or extensive diffuse infiltration of the lamina propria independent of submucosa invasion. The variation of the degree of lesions by group was investigated by analysis of variance (ANOVA).

**PCR.** Tracheal swabs were collected at the 8th, 12th, 15th, 18th, 20th and 24th week and stored in 1 ml of medium Frey. Each swab sample was then packaged in graduated tubes of 1.5ml and had their DNA extracted by the phenol-chloroform adapted from

Sambrook et al. (1989), without pre-enrichment. The quantification of DNA was performed using the spectrophotometer Biodrop Touch® (Biochrom) with values around 25-30ng/ul.

The pair of "primers" and amplification conditions for MG-F PCR followed Nascimento et al. (1993). As a positive control we used the MG - F vaccine (Ceva Animal Health, São Paulo, SP, Brazil). The PCR for MG - F was performed under the following conditions: 56µL of ultrapure water (Milli - Q), 10µL of 10X PCR buffer, 8µL MgCl<sub>2</sub> (25 mM), dNTP mix 5µL (0.25 mM each), 2µL (100pmol) of each " primer " (5'CGT GGA TAT CTT TTC TAG CAG CTG GCA C3'e 5'GTA AGT TAT CAG GCA AAT TTC T3'), 2µL (2.5 U/µL) of Taq Polymerase and 15µL DNA extracted to yield a final volume of 100µL. After amplification reaction, 10µL of each sample were homogenized with 2µL of buffer and applied in 1.5% agarose gel submerged in Tris- Borate- EDTA (TBE) 0.5X , and finally uploaded to the electrophoresis conditions based on Sambrook et al. (1989). After electrophoresis, the gel was stained in ethidium bromide and proceeded to display the "amplicons" of 524 base pairs under ultraviolet light transilluminator.

## RESULTS AND DISCUSSION

At the 8th week of age, blood samples from the chickens were negative for *Mycoplasma gallisepticum* (MG) by SAR and ELISA, being the score averages by SAR of 0 and optical density (OD) averages of 0.163; 0.172 and 0.172 for groups 1, 2 and 3 respectively. Tracheal swabs collected at the 8th and 12th week were negative for MG-F PCR (Fig.1).

Up to 12 weeks there was no significant difference among groups by SAR and ELISA (Kruskal-Wallis test,  $p > 0.05$ ). SAR reactions appeared from the 15th week in G2 and G3 (Tukey-Kramer,  $p < 0.05$ ) while by ELISA it occurred in the 18th week (Tukey-Kramer,  $p < 0.05$ ) (Table 1 and 2). Positivity in G3 by PCR occurred seven weeks after exposure and in the 15th week all contact birds were positive by PCR (Fig.1) and SAR, agreed with Kleven et al. (1981) and Avakian et al. (1988) which concluded that MG infection was transmitted during the first 4 weeks postinfection. Furthermore, Avakian et al. (1988) proved that SAR as more sensitive than ELISA and HI test during three weeks postinfection. Pakpinyo et al. (2013) detected vaccine MG-F by SAR and PCR in all birds six weeks post vaccination, ELISA's reactions were present but appear in fewer layers.

**Table 1. ELISA serologic response in OD for MG-F in chickens unvaccinated (G1), vaccinated at 8<sup>th</sup> weeks of age (G2) and vaccinated by contact (G3) according to age in weeks**

Groups	12th*	15th**	18th**	20th**	24th** week
1	0,1551±0,013 <sup>a</sup>	0,1551±0,013 <sup>a</sup>	0,1867±0,028 <sup>a</sup>	0,1428±0,06 <sup>a</sup>	0,1564±0,061 <sup>a</sup>
2	0,151±0,0312 <sup>a</sup>	0,189±0,079 <sup>b</sup>	0,4695±0,2095 <sup>b</sup>	0,455±0,203 <sup>b</sup>	0,414±0,1871 <sup>b</sup>
3	0,146±0,0189 <sup>a</sup>	0,129±0,034 <sup>b</sup>	0,555±0,2085 <sup>b</sup>	0,509±0,207 <sup>b</sup>	0,508±0,1904 <sup>b</sup>

\*ANOVA/ Kruskal-Wallis  $p < 0,05$ ; <sup>a,b</sup> different letters on the same column means significant differences.

\*\* ANOVA/ Tukey-Kramer.

**Table 2. Response by SAR for in chickens unvaccinated (G1), vaccinated at 8<sup>th</sup> weeks of age (G2) and vaccinated by contact (G3) according to age in weeks**

Groups	12th*	15th**	18th**	20th**	24th** week
1	0,05±0,2357 <sup>a</sup>	0,17±2,95 <sup>a</sup>	1,76±2,873 <sup>a</sup>	0,1±0,3162 <sup>a</sup>	0,15±0,366 <sup>a</sup>
2	4,968±3,361 <sup>a</sup>	7,81±3,69 <sup>b</sup>	7,65±3,498 <sup>b</sup>	8,25±0,2447 <sup>b</sup>	6,29±3,667 <sup>b</sup>
3	0,05±0,2236 <sup>b</sup>	8,1±3,25 <sup>b</sup>	8,5±2,415 <sup>b</sup>	9,5±1,581 <sup>b</sup>	6,16±4,446 <sup>b</sup>

\*ANOVA/Kruskal-Wallis  $p < 0.05$ ; <sup>a,b</sup> different letters on the same column means significant differences.

\*\* ANOVA/Tukey-Kramer.

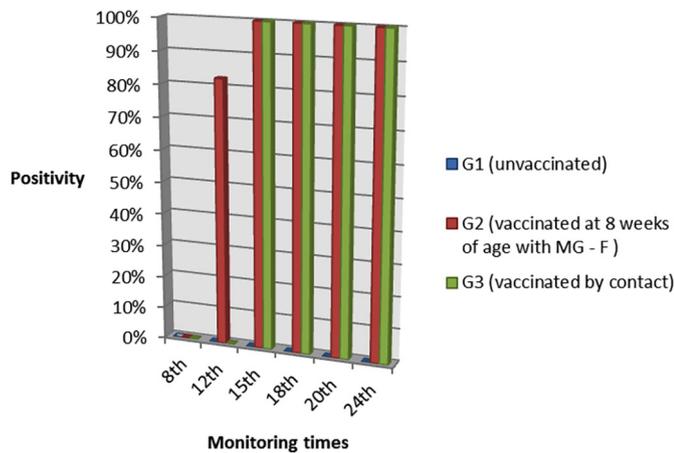


Fig.1. Experimental chickens positivity percentage by PCR at monitoring times in unvaccinated, eight week vaccinated and contact groups.

**Table 3. Mean scores of gross lesions (M) and microscopic (m) in tracheas and air sacs in chickens no vaccinated (G1), vaccinated (G2) and vaccinated by contact (G3) with F strain of *Mycoplasma gallisepticum* (MG-F), at different stages of the experiment**

Vaccination	Trachea				Air sac			
	15th week		24th week		15th week		24th week	
	M	m	M	m	M	m	M	m
G1	0,20 <sup>a</sup>	0,1 <sup>a</sup>	0,25 <sup>a</sup>	0,07 <sup>a</sup>	0,3 <sup>a</sup>	0,2 <sup>a</sup>	0,125 <sup>a</sup>	0,15 <sup>a</sup>
G2	0,6 <sup>a</sup>	0,0 <sup>a</sup>	0,64 <sup>a</sup>	1,00 <sup>a</sup>	0,91 <sup>a</sup>	0,32 <sup>a</sup>	0,71 <sup>a</sup>	0,66 <sup>b</sup>
G3	0,6 <sup>a</sup>	0,4 <sup>b</sup>	0,60 <sup>a</sup>	0,46 <sup>b</sup>	0,9 <sup>a</sup>	0,55 <sup>a</sup>	0,60 <sup>a</sup>	0,80 <sup>b</sup>

\*ANOVA/Kruskal-Wallis  $p < 0,05$ ; <sup>a,b</sup> different letters on the same column means significant differences.

The average scores for the gross and microscopic lesions by group were obtained (Table 3). There was no significant difference between the mean scores of the gross lesions of trachea and air sac. Whereas the mean scores of microscopic tissue changes appeared in the 15th week ( $p=0.1266$ ) and 24th week ( $p=0.0379$ ) in trachea and at 24th week ( $p=0.0391$ ) in air sac. Regarding trachea, G3 showed no lesions in 15th week (0.00) as compared to G1 (0.10) and G2 (0.48); lesions were observed at the 24th week (1.00) compared to G1 (0.077) and G3 (0.46). Probably G3 did not have tissue changes in the 15th week because the chicken did not receive directly the vaccination, that is, major changes occurred at the 24th week when start of local immune reactions. All birds yielded low scores throughout the experimental period, proving that MG-F was not causing significant tissue changes in the trachea and air sac. The present study agree with the reports of Levisohn et al. (1983) and Pakpinyo et al. (2013) who concluded that MG-F colonization of the tracheal tissue was accompanied by discrete changes. Pakpinyo et al. (2013) also found that MG-F caused no injuries in air sac two weeks after vaccination So the presence of low scores in this study is explained by the long time after for histopathological evaluation vaccination. Furthermore, the method of inoculation by eye drop induces less aerosaculitis than aerosol (Lin & Kleven 1984).

## CONCLUSION

SAR and PCR were able to detect the transmission of MG-F earlier than ELISA. Compared with the negative control, the

tissue reactions to the vaccine groups were more intense in the 24th week, it seems to be response to vaccination.

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