SEROLOGICAL RESPONSE OF COMMERCIAL LAYING HENS TO *Mycoplasma gallisepticum* IN POULTRY FARMS IN SÃO PAULO STATE

(REAÇÕES SOROLÓGICAS CONTRA Mycoplasma gallisepticum EM AVES DE POSTURA DE GRANJAS COMERCIAIS NO ESTADO DE SÃO PAULO)

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SUMMARY

The seroreactivity of commercial laying hens for *Mycoplasma gallisepticum* was evaluated by plate seroagglutination test (SAR) and ELISA, a screening and confirmatory test, respectively. The trial run during June and July, 2009 in poultry farms located in the Bastos region and in the city of Guatapará, SP. The Bastos region total seroreactivity for SAR was 88.2% (566/642), from which 85.5% (196/229) non-vaccinated and 89.5% (370/413) vaccinated laying hens. Whereas total seroreactivity for ELISA was 89.8% (577/642), from which 97.40% (233/229) non vaccinated and 85.7% (354/413) vaccinated laying hens. In the municipality of Guatapará, all the flocks were vaccinated against *M. gallisepticum*, and seroreactivity rates were 76.5% (108/141) for SAR and 97.20% (137/141) for ELISA. In the region of Bastos, 84.30% (193/229) of the non- vaccinated hens were simultaneously reactive to SAR and ELISA; however, there was no clinical case of infection, and this high rate of seroreactivity may be due to the diffusion of live vaccine strain (ts-11 or F), which have low pathogenicity and immunize hens against wild strains. The high rate of vaccinated hens in both regions simultaneously reactive to SAR and ELISA, 84.00% (465/554) or the 8.80% (49/554) reactive to ELISA only, as well as the absence of clinical signs show low vaccine failure and its effectiveness in protecting the flocks against chronic respiratory disease.

KEY-WORDS: Chronic respiratory disease. Commercial laying hens. Mycoplasmosis. Sorodiagnosis.

RESUMO

Avaliaram-se reações sorológicas contra *Mycoplasma gallisepticum* em aves de postura comercial no Bolsão de Bastos e no Município de Guatapará, Estado de São Paulo, no período junho-julho/2009, utilizando a soroaglutinação rápida (SAR) como triagem e o ELISA como teste confirmatório. No Bolsão de Bastos, na SAR observou-se sororreatividade total de 88,2% (566/642), sendo para aves não vacinadas contra *M. gallisepticum* 85,5% (196/229) e vacinadas 89,5% (370/413), e pelo ELISA, reatividade total 89,8% (577/642), para aves não vacinadas 97,40% (233/229) e aves vacinadas 85,7% (354/413). Em Guatapará, todas as granjas amostradas utilizavam vacinação (cepa ts-11); a proporção de reagentes na SAR foi 76,5% (108/141) e no ELISA 97,20% (137/141). No Bolsão de Bastos, onde as aves não vacinadas apresentaram elevados índices de sororreatividade simultaneamente na SAR e no ELISA, 84,30% (193/229), não foram observados casos clínicos da infecção, fato que pode ser atribuído à difusão de cepas vacinais vivas (F ou ts-11), que possuem baixa patogenicidade e imunizam as aves contra as cepas de campo. O grande percentual de aves imunizadas, nas duas regiões, que foram reagentes simultaneamente na SAR e no ELISA – 84,00% (465/554) ou reagentes somente ao ELISA – 8,80% (49/554) e a ausência de sinais clínicos indica que houve pouca falha vacinal, com a vacina protegendo-as da doença crônica respiratória.

PALAVRAS-CHAVE: Aves de postura comercial. Doença crônica respiratória. Micoplasmose. Sorodiagnóstico.

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Submitted: 26/02/2011 Accepted: 08/02/2012

INTRODUCTION

Mycoplasma gallisepticum is a major problem for the poultry industry since it is associated with chronic respiratory disease (CRD) and can be transmitted vertically by the egg. The economical losses are due to declining egg production and quality (NOORMOHAMMADI et al., 2002), as well as low hatchability, high rate of culled chicks, poor feeding efficiency, high drug costs, bacterial resistance and trade restrictions (METTIFOGO & BUIM, 2009).

The Plano Nacional de Sanidade Avícola (PNSA, National Poultry Health) provides for the official monitoring of broiler breeder (parents, grandparents and genetic nucleus farms) for *M. gallisepticum*, *M. synoviae* and/or *M. meleagridis*, following epidemiological sampling procedures and frequency. The diagnostic screening and confirmatory tests are plate seroagglutination test (SAR) and haemagglutination inhibition (HI) or ELISA, respectively, as well as isolation and typifying and/or PCR. Additionally, broilers can be tested before and during slaughtering, as well commercial laying hens at farms (BRASIL, 1994).

Although *M. gallisepticum* is not officially controlled in laying hen farms, seroepidemiological studies were performed using SAR, in Brazil. In Rio de Janeiro, Danelli et al. (1999) found 17% (11/64) seropositive non-vaccinated poultry, Mendonça et al. (2003) reported 41.7%, and Simas et al. (2008), 94% (47/50). In Rio Grande do Sul, Rauber et al. (2004) reported for chicken farms, A - 26% (7/27), B – 56.6% (34/60) and C – 18.1% (27/149). In Minas Gerais, Santos et al. (2007) found 2% (6/300) seropositive non-vaccinated poultry.

The live vaccine is recommended for commercial laying hens to reduce production losses and to prevent transmission of infection (NASCIMENTO, 2000). The use of live vaccine against *M. gallisepticum* causes weak immune response, while high titers of antibodies in ELISA indicate that the vaccinated groups are facing the challenge in the field, and it is therefore, necessary to verify the presence of clinical signs (BUTCHER, 2009; MUÑOZ et al., 2009).

Giving the economic importance of commercial laying hens in two regions of São Paulo state: the Bastos region and the city of Guatapará, as well as the lack of data regarding the presence of *M. gallisepticum* in the poultry industry, this study evaluated the occurrence of serological reactions using SAR and ELISA, as screening and confirmatory tests, respectively, as well as bird humoral response to vaccination according to production stage and age.

MATERIAL AND METHODS

Blood samples were collected from commercial laying hens in two regions of São Paulo, Bastos and Guatapará. The Bastos region is located in Nova Alta Paulista, latitude 21º55'19" S and longitude 50º44'02" W, at 445 m altitude and covers an area of 170.454 km². This region has the largest flock of laying hens and is responsible for the largest egg production in Brazil (CDA, 2009). The Bastos region was created by Resolution SAA-27, on 30/09/2003, and includes 16 other counties besides Bastos, among them Iacri, Osvaldo Cruz and Tupã. It accounts for 15% of the national flock of laying hens and produces more than 8.6 million eggs per day (SÃO PAULO, 2003). The chicken farms studied were selected from the database of the Sindicato Rural de Bastos, SP, according to the criterion whether the animals were vaccinated or not against mycoplasmosis.

The city of Guatapará is located in the Ribeirão Preto region, northeast of São Paulo, at latitude 21º29'48" S and longitude 48º02'16" W, covers an area of 412.637 km² (CDA, 2009). The main economic activity is egg production, averaging 21,000 dozen eggs per day, and ranks as the 18th Brazilian city in egg production according to the Instituto Brasileiro de Geografia e Estatística (IBGE, 2009). The database of the Cooperativa Agrícola Mombuca was used to select the chicken farms in this county.

In the Bastos region, blood samples were collected in 10 chicken farms, four lots per farm, on average 16 birds per lot. The animals were classified according to age (weeks) and production stage, beginning, peak and end of egg production. In Guatapará, 160 blood samples were collected in each one of the 10 farms.

Blood samples were collected by venipuncture of the humeral vein, using a 3-mL disposable syringe and needle 21 x 0.7 mm. Every 3-mL blood sample was labeled according to lot and farm to which it belonged. The procedures were approved by the Ethics Committee for Animal Experimentation of the Instituto Biológico (CETEA-IB), registered under protocol CETEA 77/09, approved on March 18, 2009.

After blood coagulation and serum separation, the SAR test was performed. An aliquot of each serum sample was individually stored in *Eppendorf* vials and frozen at -20ºC to be used for ELISA.

The methodology used to perform SAR and ELISA is recommended by MAPA (Brasil, 1994). The SAR antigen used was Myco-Galli Teste®, BIOVET, consisting of a suspension of inactivated *M. gallisepticum* strain S-6. All vials belonged to the same 869/08 lot. The ELISA was performed using a kit to detect antibodies against *M. gallisepticum* Flock Chek Idexx®, following manufacturer guidelines. The ELISA cutoff to determine whether a sample was reagent or not, geometric mean titer (GMT) as well as the other statistical variables, coefficient of variance (CV), standard deviation (SD), maximum and minimum values were calculated by the software xCheck 3.3 (Idexx®).

Statistical differences between seroreactivity levels of SAR and ELISA for vaccinated and non-vaccinated animals, according to production stage (age) were calculated by Chi-square test, at 5%.

The GMT values of the ELISA test for *M. gallisepticum* in vaccinated birds, according to production stage and age, for both regions Bastos and Guatapará, SP, were compared by Tukey test, at 5%.

RESULTS AND DISCUSSION

The Bastos region had a high rate of birds reagents to SAR and ELISA, respectively, 88.2% (566/642) and 89.8% (577/642). The vaccinated lots had 89.5% (370/413) seroreactivity to SAR, whereas among the non-vaccinated 97.4% (223/229) were reactive to ELISA. The birds were vaccinated with live strains F or ts-11, and seroreactivity of these lots varied from 50 to 100% for SAR and from 31.25 to 100% for ELISA. For non-vaccinated birds, seroreactivity varied from 0 to 100 % for SAR and from 78.95 to 100 % for ELISA.

In Guatapará where all the vaccinated chicken received the live strain ts-11, there was a high rate of birds reactive to SAR and ELISA, 76.5% (108/141) and 97.2% (137/141), respectively. The seroreactivity of each lot varied from 50 to 100% and from 80 to 100% for SAR and ELISA, respectively.

It was important to evaluate the response of both tests together, screening SAR and confirmatory ELISA, since SAR detects IgM and indicates seroconversion, while ELISA detects IgG and chronic infection (NASCIMENTO, 2000; BUTCHER, 2009).

In regions of intense poultry business, such as Bastos and Guatapará, where the vaccination uses live strains of low pathogenicity against mycoplasmosis, the vaccine agent is widely spread by aerosol among lots and even among farms, which may end up infecting non-vaccinated birds with the attenuated strain. In the Bastos region, 84.3% (193/229) of the non-vaccinated birds were seroreactive to SAR and ELISA simultaneously, which indicated that the majority was in the acute stage of infection; however, there were no birds with respiratory signs. These lots with non-vaccinated seroreactive birds were probably infected with the mild strain contracted from the vaccinated birds of the same farm or neighboring farms that was spread in the environment. These low pathogenic live vaccine strains F and ts-11 colonize the upper respiratory tract of birds and compete with pathogenic strains for a connection with the receptors on the tracheal epithelium (METTIFOGO & BUIM, 2009).

Considering the high number of vaccinated birds in these two regions, the humoral response of these two populations was also evaluated. A high number was reactive to both SAR and ELISA, 84% (465/554), showing that the birds had IgM and IgG antibodies or reactive to ELISA only, 8.8% (49/554), with IgG antibodies. These data indicated the humoral response expected in a population that is subjected to mass vaccination using live attenuated strain, where a large percentage of seroreactive birds can be found. Since no clinical respiratory symptoms were observed in the vaccinated birds, one can assume that spreading of the live attenuated strain protected the population against virulent field strains (METTIFOGO & BUIM, 2009). In addition, a low percentage of vaccinated birds were not reactive to both ELISA and SAR simultaneously, 3.6% (20/554), thus indicating low vaccine failure.

In both regions, mycoplasmosis control is accomplished primarily by vaccination and not biosafety measures. The farms were densely populated and very close, with no physical barrier to separate them. It is also very common to have birds of multiple ages, at different reproduction stages in the same farm, which may have contributed to the spread of the strains and high total reactivity to SAR 86.1% (674/783) and ELISA 91.2% (714/783). Ito et al. (2002) emphasized that biosafety is the single most important preventive measure to avoid introduction of *M. gallisepticum* in chicken farms, and Nascimento (2000) points out that multiple lots of different ages in the same farm act as a facilitator for emergence of intercurrent diseases and the spread of pathogens among the birds of the same lot and among lots as well.

Regardless of the production system of commercial laying hens, the egg production phase lasts the same, it starts and finishes on the 17^{th} and 72^{nd} weeks, respectively (ALVES, 2006; GARCIA & MOLINA, 2008). In order to evaluate the impact of the production stage on the seroreactivity of the vaccinated lots, the laying period was divided into early (17-35 weeks), middle (36-53 weeks) and final (from the 54^{th} week on). The Chi-squared test showed a statistically significant effect of age on the seroreactivity to *M. gallisepticum*; fewer birds aged $\geq$ 54 had IgM antibodies detected by SAR ($P = 0.0499$) and more birds had IgG antibodies by ELISA ($P = 0.0162$) (Table 1), thus showing the chronicity of infection or vaccination (METTIFOGO & BUIM, 2009).

There was a statistically significant effect of age on the seroreactivity to *M. gallisepticum* of non-vaccinated birds; more birds aged $\geq$ 54 weeks had IgM antibodies ($P < 0.001$), indicating recent infection and also chronicity by IgG ($P < 0.001$) (Table 2); however, since there were no birds with clinical respiratory symptoms, one concludes that the vaccine strain of low virulence spread stimulating the immune system (METTIFOGO & BUIM, 2009).

The geometric mean titer (GMT) of ELISA for vaccinated bird varied widely within the same lot and farm, in both regions, also standard deviation (SD), coefficient of variance (CV) and minimum and maximum values were high. The comparison of GMT values of ELISA of vaccinated lots by Tukey test at 5% showed no statistical difference regarding production phase and age ($P>0.05$) (Table 3). Taking into consideration that laying hens are vaccinated young, the serological reactions after live vaccine did not result in high titers of ELISA, the presence of high titers indicate field challenge, however, as there were no clinical signs of CRD, one can hypothesize that his humoral immune response is due to infection by attenuated vaccine strain or low virulence field (BUTCHER, 2009; MUÑOZ et al., 2009).

Since vaccination against *M. gallissepticum* is widely spread in commercial laying birds, it was difficult to find non-vaccinated animals and therefore, it was not possible to apply Tukey test to compare GMT of different ages in the Bastos region.

SAR is a screening test with low specificity, which is why false-positive results may occur. This fact has contributed to a low degree of certainty when diagnosing mycoplasmosis, especially when it is used

Table 1 – Seroreactivity of hens vaccinated against *M. gallisepticum* determined by SAR and ELISA, according to production stage and age, in the Bastos region and Guatapará, SP (São Paulo, 2009).

Production stage (age)	SAR vaccinated		ELISA vaccinated	
	Reactive % (subtotal)	Non reactive % (subtotal)	Reactive % (subtotal)	Non reactive % (subtotal)
Beginning (17-35 weeks)	80.30 (98/122)	19.70 (24/122)	81.15 (99/122)	18.85 (23/122)
Middle (36-53 weeks)	91 (111/122)	9 (11/122)	89.85 (124/138)	10.15 (14/138)
Final (> 54 weeks)	69.70 (269/310)	30.30 (41/310)	90.80 (267/294)	9.2 (27/294)
Total	86.3 (478/554)	13.7 (76/554)	88.40 (490/554)	11.60 (64/554)
Chi-squared	(P = 0.0499)		(P = 0.0162)	

Table 2 – Seroreactivity of hens not vaccinated against *M. gallisepticum* determined by SAR, according to production stage and age, in the Bastos region (São Paulo, 2009).

Production stage (age)	SAR non-vaccinated		ELISA non-vaccinated	
	Reactive % (subtotal)	Non reactive % (subtotal)	Reactive % (subtotal)	Non reactive % (subtotal)
Beginning (17-35 weeks)	50.75 (34/67)	49.25 (33/67)	92.50 (62/67)	7.50 (5/67)
Middle (36-53 weeks)	100.00 (48/48)	0	97.90 (47/48)	2.10 (1/48)
Final (> 54 weeks)	100.00 (114/114)	0	100 (114/114)	0
Total	85.50 (196/229)	14.50 (33/229)	97.40 (223/229)	2.60 (6/229)
Chi-squared	(P < 0.001)		(P < 0.001)	

Table 3 – Comparison of GMT of vaccinated birds determined by ELISA for *M. gallisepticum*, according to production stage and age, in the Bastos region and Guatapará, SP, by Tukey test (São Paulo, 2009).

Production stage (age)	Number of lots	Mean GMT*	SD	CV minimum	CV maximum	GMT minimum	GMT maximum	Confidence interval 95%
beginning (17-35 weeks)	8	3,645.0a	2,736.1	25.2 %	118.1%	1,417	8,413	1,357.2 to 5,932.8
middle (36-53 weeks)	8	7,927.6a	5,394.6	22.1 %	63.0%	741	15,682	3,416.9 to 12,438
Final (>54 weeks)	20	6,810.3a	4,218.7	17.4 %	81.2%	986	19,359	4,835.9 to 8,784.7

* Means followed by the same letter in the rows are not significantly different (P>0.05)

alone (BRASIL, 1994; MENDONÇA et al., 2003; MENDONÇA et al., 2004; BUCHALA et al., 2006; CARDOSO et al., 2006). The false-positive reactions can also be attributed to the presence of globulin and other serum components in the growth medium used in the mycoplasma culture for production of antigen for SAR test (AHMAD et al., 1988). It is also known that there are significant differences in sensitivity and specificity of SAR antigens (ROSALES, 1999) and variability among lots of the same manufacturer and between diagnosis laboratories (METTIFOGO & BUIM, 2009). However, we used antigens from the same lot and manufacturer for all SAR tests. The possibility of false-positive by contaminated serum was also (METTIFOGO & BUIM, 2009) ruled out since we used sterile new disposable syringes and needles for each bird and serology was performed as soon as the serum was separated from the blood.

The live vaccine strains ts-11 and F were used in the Bastos region, while in Guatapará only the strain ts-11 was used. On way to differentiate infection caused by field strains or by vaccine strains is to perform the multiplex PCR, which assists the differential diagnosis of isolated and epidemiological studies (METTIFOGO & BUIM, 2009), and such study can be performed in a later stage. The high levels of seroreactivity to SAR and ELISA associated with the lack of clinical symptoms suggest the occurrence of attenuated vaccine strains or field of low virulence. Only an epidemiological study isolating *M. gallisepticum* to perform molecular characterization and differentiation can determine the strains that are stimulating the humoral response of the hens.

CONCLUSION

The high percentage of hens vaccinated with live attenuated strain of *M. galissepticum* reactive to both ELISA and SAR simultaneously, associated with the absence of clinical symptoms indicate that there was low vaccine failure in these regions, and that vaccination stimulated the immune response and is effectively protecting the animals against chronic respiratory disease.

ACKNOWLEDGMENTS

Thanks are due to the veterinarian Wagner Watanabe, of Associação Paulista de Avicultura, and to the technician Cristóvão Altero, of EDA in Tupã, for helping collecting the samples. To BIOVET, for donating the SAR antigen for *M. gallisepticum*, and to APA, for donating the kits ELISA MG.

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