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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Effects of F-Strain *Mycoplasma gallisepticum* Inoculation at Twelve Weeks of Age on Serum Vitellogenin Concentrations in Commercial Egg Laying Hens[1,2]

E.D. Peebles[3], M.R. Burnham[3], S.L. Branton[4], K.O. Willeford[5] and P.D. Gerard[6]
[3]Department of Poultry Science, [5]Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS, 39762, USA
[4]Poultry Research Unit, Agricultural Research Service, USDA, Mississippi State, MS, 39762, USA
[6]Department of Mathematical Sciences, Clemson University, Clemson, SC 29634, USA

Abstract: The objective for this study was to determine if circulating vitellogenin (VTG) concentrations throughout a complete egg laying cycle in commercial layers are affected by a pre-lay inoculation of F-strain *Mycoplasma gallisepticum* (FMG). At 20, 28, 32, 36, 40, 44, 48 and 52 wk of age, blood was collected and serum was extracted for analysis of VTG concentration in layers that had either been sham-inoculated (control) or inoculated with FMG at 12 wk of age. Ten birds were housed in each of 8 biological isolation units with 4 replicate units per treatment. Each wk, sera from 4 independently tagged birds in each unit were pooled for analysis. The molecular weight of VTG in the pooled serum samples from each unit was estimated by comparison with the electrophoretic mobilities of proteins of known molecular weight in polyacrylamide gels. Additional comparisons were made using serum from estrogenized roosters known to have elevated VTG concentrations. Electrophoretic image analysis and VTG quantitation were determined via a multi-image analyzer and associated software. The effects of bird age, FMG treatment and their interactions on VTG concentration were determined. There was a significant main effect due to hen age on serum VTG concentration; however, FMG treatment had no significant effect on serum VTG concentration throughout the lay cycle. Therefore, it was concluded that the effects exerted by FMG on layer performance and egg yolk composition, as noted in previous research, may not be attributed to the influences of FMG on serum VTG concentrations.

Key words: Electrophoresis, layer, *Mycoplasma gallisepticum*, serum, vitellogenin

INTRODUCTION

The hepatic production of vitellogenin (VTG), a serum lipoprotein that is specific to laying hens and which acts as a transporter of yolk granule proteins to the ovary, is stimulated by the secretion of 17beta-estradiol from the thecal cells of hens (Bergink *et al*., 1974; Deeley *et al*., 1975; Christmann *et al*., 1977; Yamamura *et al*., 1995). The circulating concentrations of VTG in roosters (4 to 8 ng/mL) can be increased by the administration of exogenous estrogen. Exogenous estrogen has been found to increase VTG in roosters to concentrations that approximate those of laying hens (10 to 25 mg/ml; Blue and Williams, 1981). After its formation as a polypeptide in the liver, VTG is phosphorylated, glycosylated, associated with lipid and then secreted into the circulatory system as a phosphoglycoprotein dimer with a molecular weight of approximately 500 kDa (Deeley *et al*., 1975). Upon its transport to the ovary, the translocation of VTG across the plasma membranes of oocytes is mediated by membrane receptors located in coated vesicles (Shen *et al*., 1993). The VTG is subsequently cleaved into 2 major yolk phospho-proteins, lipovitellin and phosvitin (Tata, 1976), which are deposited into developing oocytes.

The performance and egg (Burnham *et al*., 2002a), reproductive organ (Burnham *et al*., 2002b), blood (Burnham *et al*., 2003a), egg yolk (Burnham *et al*., 2003b) and lipoprotein characteristics (Burnham *et al*., 2003c) of the layers used in this study have previously been reported. Egg production (EP) was depressed and onset of lay was delayed in these birds when inoculated at 12 wk of age with the F-strain of *Mycoplasma gallisepticum* (FMG). Furthermore, these birds experienced a reduction in mature ovarian follicle numbers, increased yolk linoleic acid content, and decreases in total yolk lipid at 22 wk and yolk cholesterol at 28 wk of age. Plasma protein and serum triglyceride levels were altered but the physical properties or relative concentrations of their circulating lipoproteins were not affected by FMG. Peebles *et al*. (2006) have more recently reported that the timing of an S6 strain of *Mycoplasma gallisepticum* inoculation significantly alters subsequent serum calcium concentrations. These results suggest that because serum calcium concentrations may also be influenced by estrogen

Corresponding Author: E.D. Peebles, Department of Poultry Science, Mississippi State University, Mississippi State, Mississippi 39762 - USA

(Etches, 1987), that serum calcium and VTG might respond similarly and through associated pathways to mycoplasmal infections.

The objective of the current study was to determine possible changes in serum VTG concentrations associated with changes in performance throughout a complete egg laying cycle in commercial layers inoculated with FMG at 12 wk of age. Determinations of the effects of FMG on serum VTG in association with various production characteristics, as noted in earlier related studies, may provide vital information as to the physiological mechanisms behind the previously observed alterations in the performance of FMG-infected hen.

MATERIALS AND METHODS

Data collection: Detailed descriptions of bird housing, management, FMG inoculation and mycoplasma detection are provided by Burnham *et al.* (2002a). The trial was conducted under an approved USDA animal care and use protocol. At 20, 28, 32, 36, 40, 44, 48 and 52 wk of age blood was collected from the same hens following an overnight fast. Individual blood samples from four tagged hens in each of four biological isolation units, within sham-inoculated control and FMG-inoculated treatment groups, were centrifuged for extraction of serum. Individual serum samples from each of the four replicate units per treatment were pooled prior to analysis. High speed subnatant fluid from the blood of estrogenized roosters known to be highly concentrated with VTG was used as a comparison in this study. Estrogenized rooster serum and the serum from FMG-free and FMG-inoculated hens were diluted 1:20 with sample buffer.

Electrophoretic analysis of serum vitellogenin: Dilutions of serum (10 µL) were analyzed in vertical slab gels using the MiniProtean III Power Pac 300 (Bio-Rad Laboratories, Hercules, CA) discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) system (Laemmli, 1970). Separating gels (7.5%) and stacking gels (5%) were prepared from a stock solution in which the ratio of acrylamide to bisacrylamide was 30:0.8 by weight. The final composition of the sample buffer was 10 mM Tris, pH 6.8, 1 mM EDTA, 1% (wt/vol) SDS, 10% (wt/vol) glycerol and 0.001% (wt/vol) bromphenol blue. Separating gel slabs (14 cm long and 0.15 cm thick) were formed between glass plates and electrophoresis was conducted following the description of Studier (1973). Electrophoresis was carried out at 50 V until the leading edge entered the separating gel (approximately 1 h) and then at 100 V until the leading edge almost reached the bottom of the gel (approximately 7 h). After electrophoresis, the slabs were fixed in 12% (wt/vol) trichloroacetic acid for 20 min, stained in 0.2% (wt/vol) Coomassie brilliant blue R250 dissolved in methanol-water (1:1) and destained in 10% (vol/vol) acetic acid (Wallace and Selman, 1985; Wallace and Begovac, 1985). On the gel for each bird age specified, one standard (Lane 1), one estrogenized rooster serum sample (Lane 2), four control replicate serum samples (Lanes 3-6) and four replicate FMG-treatment serum samples (Lanes 7-10) were analyzed. The molecular weight of VTG in the serum samples was estimated by comparing their electrophoretic mobilities in polyacrylamide gels with the mobilities of proteins of known molecular weight and additionally compared with the VTG-induced serum. Image analysis and quantitation of serum VTG (band intensity) (ODu x mm) were assessed with a Fluor-S Multi-Imager with Quantity One software (Bio-Rad Laboratories, Hercules, CA). Broad spectrum molecular mass markers were used to estimate the molecular weight of VTG. Marker proteins and their respective molecular weights (kDa) were ribonuclease A, 14; deoxyribonuclease, 31; ovalbumin, 43; catalase, 58; bovine serum albumin, 66; phosphorylase b, 94; beta-galactosidase, 116.2; myosin 144 heavy chain, 200, and human apolipoprotein B, 250.

Statistical analysis: A completely randomized experimental design and repeated measures data analysis were utilized. In the event of significant age or treatment main effects or their interaction, mean VTG band intensities were compared by least-squares means (Steel and Torrie, 1980). Data analysis was accomplished through the use of the MIXED procedure of SAS (2000). Statements of significance were based on $P \leq 0.05$.

RESULTS AND DISCUSSION

Burnham *et al.* (2002a) reported that EP and the egg characteristics of commercial egg-laying hens inoculated with FMG at 12 wk of age were significantly different from un-inoculated controls. The effects of the pre-lay inoculation of FMG on EP included a delay in the onset of lay by 1 wk and a significant decrease in the total number of eggs laid. Furthermore, associated alterations in the liver (Burnham *et al.*, 2002b), reproductive organ (Burnham *et al.*, 2002b), blood (Burnham *et al.*, 2003a) and yolk lipid (Burnham *et al.*, 2003b) characteristics of those hens were also shown to occur in response to the pre-lay inoculation of FMG at 12 wk of age. Blood characteristics changes were more specifically shown to include plasma protein and serum triglyceride concentrations (Burnham *et al.*, 2003a).

Sekimoto *et al.* (1990) demonstrated that a decrease in EP induced by the deprivation of food and water to laying hens was closely related to a decrease in VTG synthesis and that a recovery in EP was coupled with increases in ovary and oviduct weights as well as circulating VTG concentrations. An SDS-PAGE image analysis of VTG on wk 28 in the current study is provided in Fig. 1. Trace quantitation resulted in mean VTG levels of 3.32, 3.05

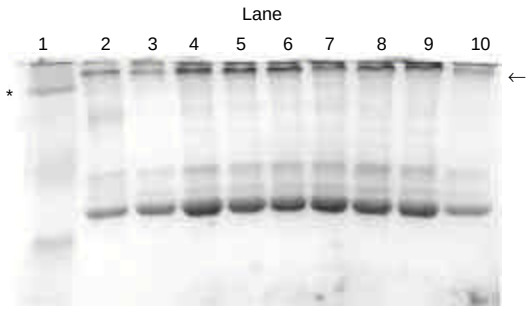

Fig. 1: Example of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) image analysis (wk 28) and quantitation of serum vitellogenin (indicated by " ← "). Scanned with a Flour-S Multi-Imager and accessed with Quantity One software (BIORAD). Lane 1 = Kaleidoscope pre-stained standards [including myosin 144 heavy chain (200 kDa)*], Lane 2 =estrogenized rooster serum, Lanes 3-6 = F-strain Mycoplasma gallisepticum (FMG)-free layer hen serum and Lanes 7-10 = FMG-inoculated layer hen serum

and 3.04 Odu x mm in sera from control hens, FMG-inoculated hens and estrogenized roosters, respectively. Nevertheless, there were no significant main or interactive (age x treatment) effects of the wk 12 FMG inoculation treatment on serum VTG concentration. Burnham *et al*. (2003c) likewise noted that the diameters of circulating very low density lipoprotein (VLDL) particles and the concentration of serum cholesterol belonging to each of the circulating populations of VLDL, low density lipoprotein and high density lipoprotein particles in commercial layers were not affected by an FMG inoculation at 12 wk of age. Therefore, VTG concentration as well the physical characteristics of VLDL particles or the relative distribution of serum cholesterol among the 3 lipoprotein classes were not affected by the inoculation of FMG. It is also important to note that the associated decreases in EP and circulating VTG concentrations described by Sekimoto *et al*. (1990) were associated with feed and water deprivation for 5 and 2 d, respectively, whereas the birds in the current study only experienced an overnight (12 h) fast before being bled.

Nevertheless, significance ($P \leq 0.002$) was established for a bird age main effect on serum VTG (Table 1). Serum VTG concentrations significantly increased in birds between 20 and 28 wk of age. This was followed by a significant decrease between 28 and 36 wk and another significant increase between 36 and 52 wk of age. Among various factors that can influence circulating VTG levels over the production period in layers, age-related changes in VTG are known to be influenced by changes in the circulating levels of estradiol, as Jost *et al*. (1991) have shown that estradiol is capable of down-regulating the binding activity of an avian VTG gene repressor.

Table 1: Mean relative intensities of serum vitellogenin bands isolated by SDS-PAGE at 20, 28, 32, 36, 40, 44, 48 and 52 wk of laying hen age

Age (wk)	Vitellogenin (ODu x mm)[1,2]
20	2.67^{cd}
28	3.69^{ab}
32	3.41^{abc}
36	2.61^{cd}
40	2.51^{d}
44	2.33^{d}
48	2.98^{bcd}
52	3.97^{a}

$^{a-d}$Means among ages with no common superscript differ significantly ($P \leq 0.05$). [1]Pooled SEM = 0.307. [2]N = 9.

Conclusion: The estimation of serum VTG by the methods used in this study suggests that the effects of an FMG-inoculation at 12 wk of age on layer performance are not mediated through alterations in the specific quantity of VTG, as well as the physical characteristics of lipoprotein particles, nor the relative concentrations of the 3 classes of lipoproteins in the circulation. However, because birds in the current study were housed in biological isolation units, these results, therefore, do not preclude the possibility that these yolk precursors may be affected differently in FMG-infected birds that are housed in stressful facilities or environments. It is suggested that the response of serum VTG concentrations in layers to the pre-lay inoculation of other *Mycoplasma gallisepticum* vaccine strains should also be evaluated.

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