

Serum ovotransferrin as a biomarker of inflammatory diseases in chickens

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ABSTRACT Infectious and metabolic disorders are common in poultry and cause stress, poor performance, and mortality that results in considerable economic loss. Identifying the nature of stress in chickens will assist the development of appropriate measures to improve health and welfare. Acute phase proteins are hepatic proteins, the blood concentrations of which change significantly in the event of many health problems including inflammation and physical injuries. Thus, acute phase proteins are used as nonspecific diagnostic markers for various health disorders. Our previous studies showed that serum ovotransferrin (OVT) is an acute phase protein in chickens. Therefore, in the present study, we investigated whether OVT concentration can be a marker of physiological stress using sera from chickens with different infectious and metabolic disorders. A competitive

enzyme immunoassay was developed to measure serum OVT concentrations. The results show that with experimentally induced pulmonary hypertension syndrome and tibial dyschondroplasia, there were no significant changes in OVT levels compared with matched controls. In contrast, when chickens were infected with microbes such as the bacterium *Escherichia coli*, or protozoan parasites such as *Eimeria maxima* and *Eimeria tenella*, there was a significant increase in the levels of OVT in the serum. Chickens with spontaneous autoimmune vitiligo also showed a significant increase in blood OVT levels. These studies suggest that blood OVT concentration is modulated under inflammatory and microbial stress and can therefore be used as a diagnostic marker of infection and inflammation in chickens.

Key words: chicken, ovotransferrin, biomarker, inflammation, metabolic disorder

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INTRODUCTION

Infectious and metabolic disorders are common in poultry and affect well-being, cause stress, reduce performance, and increase mortality. Although pathogens such as bacteria, viruses, and parasites cause inflammation and infection, metabolic problems in poultry are related to genetics, management, and environmental challenges. In either case, identifying the cause of health problems utilizing diagnostic or prognostic markers should allow measures to improve poultry health and welfare. Therefore, diagnostic or prognostic markers are useful to predict the existence of health problems. The acute phase response is the innate reaction of an organism to injurious stimuli, which can cause transitional as well as protracted changes in the levels of certain hormones, cytokines, metabolites, proteins, and peptide profiles of the blood. Changes in these fac-

tors are presumed to restore physiological homeostasis and can be predictors of health problems (Gabay and Kushner, 1999; Suffredini et al., 1999; Eckersall, 2004). The acute phase proteins (**APP**) are serum or plasma proteins of hepatic origin, the concentrations of which show significant changes during microbial infections and physical trauma (Baumann and Gauldie, 1994; Gabay and Kushner, 1999; Suffredini et al., 1999; Gruys et al., 2006). Although APP are not diagnostic of any specific disease, they are useful markers of systemic activation of any inflammatory process. Acute phase proteins have been identified in many agricultural animals including poultry and have been used to assess their health status (Chamanza et al., 1999; Holt and Gast, 2000; Juul-Madsen et al., 2003; Eckersall, 2004; Murata et al., 2004; Petersen et al., 2004; Gruys et al., 2006). In previous studies, we showed that ovotransferrin (**OVT**) or conalbumin acts as an APP in chickens, the level of which is significantly elevated under chemical-, bacterial-, and viral-induced inflammation (Xie et al., 2002a,b). However, it is not known whether OVT can be useful as a general marker of physiological stress caused by various infectious and metabolic disorders.

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This report deals with the development of an indirect competitive enzyme immunoassay (EIA) to measure OVT levels in chicken blood under different metabolic- and infection-induced problems.

MATERIALS AND METHODS

Affinity-purified anti-chicken serum transferrin antibody (AI-AG8240) was purchased from Accurate Chemical Company (Westbury, NY). Protein molecular weight standard (Fermentas, Hanover, MD), 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium alkaline phosphatase substrates (Zymed Laboratories Inc., South San Francisco, CA), EIA plates, BD Bioscience Vacutainer serum tubes (VWR International Inc., West Chester, PA), and polyvinylidene fluoride membranes (BioRad, Hercules, CA) were purchased from respective vendors. All other chemicals including purified chicken OVT that is substantially iron-free (conalbumin, C0755), fish gelatin, and goat anti-rabbit IgG antibody alkaline phosphatase conjugate were purchased from Sigma-Aldrich (St. Louis, MO).

Serum Collection

All animal procedures were approved by the University of Arkansas Institutional Animal Use and Care Committee. Blood was collected from chickens with different clinical conditions along with their age-matched controls using Vacutainer tubes and was allowed to clot for 2 to 3 h before centrifugation at $250 \times g$ for 10 min after which the supernatant serum was stored at -20°C . For experimental infection or induction of diseases, the chicks were maintained in Petersime batteries (Petersime Incubator Co., Gettysburg, OH) or floor pens, fed NRC-specified diets (NRC, 1994), and given water ad libitum. In an experimental group, sham-treated birds were used as controls. Sera from 12-wk-old male turkeys, 10-wk-old pheasants, 9-wk-old quails, and 40-wk-old laying hens were collected by wing vein bleeding.

Disease Conditions

Escherichia coli Infection. Seven-day-old male broiler chicks were inoculated in the left thoracic air sac with a nonmotile lactose-negative serotype O2 strain of *E. coli* containing approximately 6×10^4 cfu of bacteria/100 μL of tryptose phosphate broth as described previously (Huff et al., 2006). The control chicks received identical volumes of tryptose phosphate broth only. The effect of infection was monitored by recording mortality. Serum was obtained from surviving challenged chicks and their matched controls at 3 wk of age.

Eimeria Infection. Serum samples were selected from 2 groups of chickens infected with 2 different species of *Eimeria*. In the first group, 9-d-old male chicks were orally challenged with approximately 10^5 oocysts of *Eimeria maxima* per bird, mixed in feed. Serum was

collected 7 d after challenge when the chickens were killed to ascertain infection (Chapman et al., 2004). The infected chicks showed the presence of the parasites discernible microscopically. In the second group, 23-d-old specific-pathogen-free chickens were orally dosed with 4.6×10^4 *Eimeria tenella* oocysts per bird according to Korver et al. (1997), the blood was collected 5 and 10 d later, and the serum was frozen. For both treatment groups, matched controls were used to obtain blood.

Pulmonary Hypertension Syndrome. Pulmonary hypertension syndrome (PHS) was induced by subjecting 1-d-old chicks to a hypobaric atmosphere that simulated a height of 2,900 m (Balog et al., 2000; Pavlidis et al., 2007). The control chicks were maintained at the equivalent altitude of 390 m until 6 wk of age. Pulmonary hypertension syndrome was confirmed by the presence of pericardial fluid or an accumulation of abdominal fluid (Balog et al., 2000).

Experimentally Induced and Naturally Occurring Tibial Dyschondroplasia. Tibial dyschondroplasia (TD) was induced by feeding thiram as described by Rath et al. (2004). Feed was withdrawn when chicks were 7 d old and the chicks were then fed diets containing 100 mg/kg of thiram to induce TD. The controls received feed without thiram. Forty-eight hours later, birds in both groups received the regular diets until the end of the experiments on d 16 when the birds were bled and killed to score the incidence and severity of TD. More than 90% of birds fed thiram developed TD using this protocol (Rath et al., 2007). The presence of TD was ascertained by an irregularly broadened growth plate at least twice the width of a normal growth plate (Rath et al., 2007). Blood was also collected by random sampling from 6-wk-old birds and identified for the presence of spontaneous TD on necropsy.

Chickens with Autoimmune Vitiligo. Autoimmune vitiligo-prone Smyth line chickens and the parental Brown line control chickens were raised in floor pens through 13 wk of age at a density of 12 birds per pen. At about 10 wk of age, 7 out of 22 Smyth line chickens developed vitiligo-like changes indicated by the loss of brown pigmentation in growing-regenerating feathers (Wang and Erf, 2004; Wick et al., 2006). At 13 wk of age, blood samples were collected from vitiliginous Smyth line chickens via the wing vein. Blood collected from age-matched Smyth line birds that did not develop vitiligo and parental brown line birds was used as controls.

EIA

Ninety-six-well EIA plates were coated with 100 μL of OVT at a concentration of 10 $\mu\text{g}/\text{mL}$ of 0.2 M sodium carbonate-bicarbonate buffer, pH 9.4, for at least 24 h. The wells were washed once with Tris-buffered saline (TBS, 100 mM sodium chloride, 20 mM Tris HCl, pH 7.2) then blocked with 1% fish gelatin in TBS for at least 2 h. Fifty microliters of freshly made chicken OVT standards, 2-fold diluted serially from 64 to 0.25

$\mu\text{g/mL}$ in TBS, was added to the wells. The control wells received TBS diluent only. Serum samples were freshly diluted 1:100 in TBS and added to the sample wells. Affinity-purified rabbit anti-chicken serum transferrin antibody, diluted to an approximate concentration of 1 $\mu\text{g/mL}$ of TBS, was added at a volume of 50 μL to all the wells. After 2.5 to 3 h of incubation at room temperature with constant rocking, the wells were washed 4 times with TBS containing 0.05% Tween-20 followed by the addition of 1:5,000 dilute goat anti-rabbit IgG-alkaline phosphatase conjugate in TBS at a volume of 100 μL to each well. After 1 h of incubation by constant rocking, the solution was removed and the wells were washed 4 times successively as above with TBS containing 0.05% Tween-20. The residual enzyme activity was developed by the addition of p-nitrophenyl phosphatase substrate (1 mg/mL of Tris buffer, pH 9.5) and incubating 15 to 30 min at 37°C. The reaction was stopped with an equal volume of 1 N NaOH and the optical density (OD) was read at 405 nm with a Spectramax plate reader (Molecular Devices Corp., Sunnyvale, CA). The percentage of OD for each OVT concentration was calculated relative to the values in the control wells that did not receive OVT. A standard curve was plotted describing the relationship between percentage of OD and the concentration of OVT using log plot. All assays were performed in triplicate and the average values were used for calculations. The percentage of values in the sample wells was similarly calculated for test sera and the OVT concentrations in the sample were calculated using the equation obtained with the standard curve.

Optimization and Assay Variation. Initial optimization of the assay with respect to primary and secondary antibody dilutions was done using a general guideline (CDER, 2001; NCGC, 2008). Intraassay variation was calculated using a pooled sample of chicken serum differentially diluted (1:100, 1:200, and 1:400) and assayed in triplicate. Interassay variation was calculated using serum concentration values of OVT using control sera assayed at different times over a period of 3 to 4 mo. The SD of the sample was divided by the average mean from at least 5 different assays and multiplied by 100 to obtain percentage of CV. Percentage of recovery was calculated using 0.5 and 1 mg of OVT spiked, pooled chicken sera that were diluted 100-fold like the nonspiked samples and assayed in triplicate.

Cross-Species Reactivity. Serum samples of pheasant, quail, and turkeys along with young broiler chickens and 40-wk-old hens were diluted as above and subjected to EIA to determine the cross-species reactivity of the assay. Also, a Western blot was performed using 10% SDS gel electrophoresis of 2.5 μL of sera followed by the transfer of the proteins to polyvinylidene fluoride membrane. The procedures were essentially as per Harlow and Lane (1988). The membrane was blocked, probed with the anti-chicken transferrin antibody diluted as above, followed by probing with the secondary goat anti-rabbit IgG-alkaline phosphatase conjugate. The

blot was developed using 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium as substrate.

Statistics. Quantitative results were expressed as mean \pm SEM, analyzed using Student's *t*-test with SAS statistical software (SAS Institute, 2004). Differences were considered significant at $P \leq 0.05$.

RESULTS

EIA

The results of a typical assay using commercial OVT as standard are shown in Figure 1. The coefficient of interassay variation was 11.2% based on a calculation of 6 assays run at different times. Using different dilutions of the same sample in a typical assay, the variation was 8.7%. The percentage recovery of exogenously spiked OVT was $95 \pm 6\%$. The assay appeared limited with respect to cross-species reactivity, failing to measure pheasant, quail, and turkey serum OVT (Figure 2). In Western blot, the antibody, however, showed cross-species recognition of corresponding OVT bands with variable intensities (Figure 3).

OVT Concentration

Comparative results of serum OVT concentrations indicate significant elevation in chickens infected with different pathogens (Table 1). All chickens exposed to either bacterial or parasitic pathogens showed significantly elevated OVT concentrations compared with their matched controls. However, chickens with PHS and TD showed no significant change in OVT levels compared with their matched controls. By contrast, Smyth line chickens with autoimmune vitiligo, but not their nonaffected hatch mates or the parental Brown line chickens, showed a significant elevation in the serum OVT levels (Table 1). The OVT values between control Smyth line and parental Brown line chickens were not significantly different ($P = 0.08$).

DISCUSSION

Transferrins are closely related iron-binding glycoproteins that occur in birds as serotransferrin produced by the liver and OVT produced by the oviduct (Superti et al., 2007). Both serum transferrin and OVT are identical with some glycosylation differences (Thibodeau et al., 1978; Baker et al., 2002; Lambert et al., 2005). Thus, the measurements of OVT have been done using nonspecific antihuman lactoferrin antibodies (Giansanti et al., 2007) and chicken OVT specific monoclonal and polyclonal antibodies (Panheleux et al., 2000; Xie et al., 2002b). In the later assay, an affinity-purified polyclonal antitransferrin IgG was coated to the wells as capture antibody for OVT and developed a competition immunoassay. A competition between biotinylated OVT and free OVT to bind to the antibody was the basis of the assay (Xie et al., 2002b). In the current study, we de-

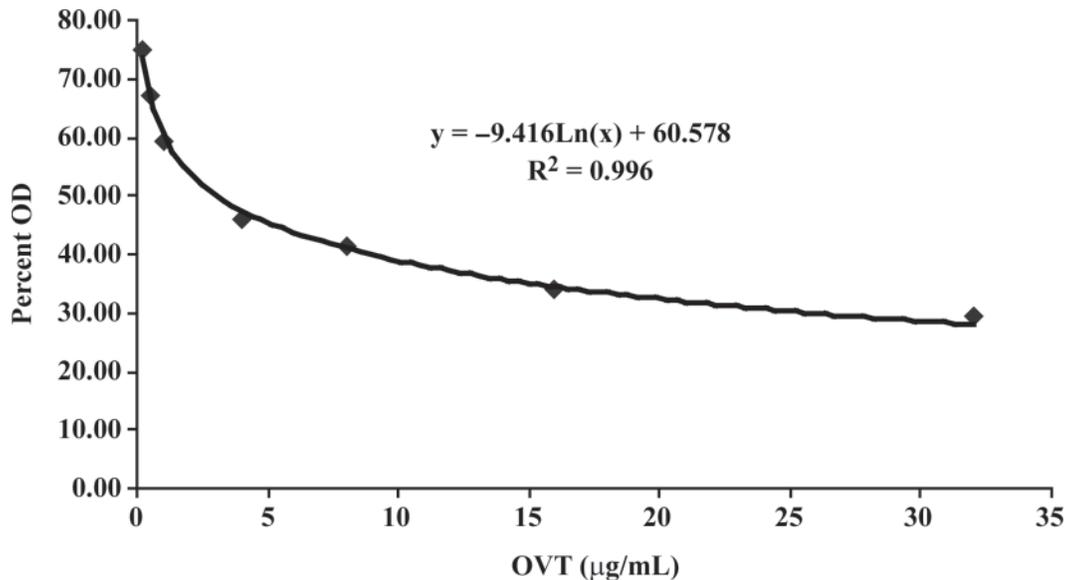


Figure 1. A typical standard curve of ovotransferrin assayed using competition immunoassay. Percentage of optical density (OD) in wells containing different concentrations of ovotransferrin (OVT) was calculated relative to the control wells that received only diluent without any OVT. The results were plotted as a log plot.

veloped a relatively simple immunometric assay based on the binding of the affinity-purified antitransferrin antibody to the OVT-coated wells in the presence of competing amounts of OVT in the solution. This procedure eliminates a relatively cumbersome OVT labeling procedure and its quality control. Using this method, the blood OVT levels in normal chicken sera showed values comparable to the earlier measurements (Xie et al., 2002b). With the intent of applying this assay to other avian species, we used serum samples from pheasant, quail, and the turkey to measure their OVT concentrations. The assay appears unsuitable to measure substantial OVT concentrations in the sera of all 3 species, although by Western blot, the antibody recognized corresponding transferrin bands with variable intensities. These results were particularly intriguing, at least, with respect to turkey transferrin, which has been shown to bear over 90% sequence identity with

its chicken homolog (Ciuraszkiewicz et al., 2006). Our attempts to resolve the issue by manipulating turkey serum to identify and eliminate interfering factors has not been successful.

Based on earlier observations from our laboratory and others, the elevation of OVT in the blood of birds suffering physiological trauma such as bacteria-, virus-, and chemical-induced acute inflammation suggested that OVT is an APP in birds (Hallquist and Klasing, 1994; Xie et al., 2002a,b). The current results using sera from both *E. coli*- and *Eimeria*-infected chickens also showed similar trends. However, it was not known whether changes in serum OVT concentrations would occur in birds with metabolic diseases. We hypothesized that OVT levels may be elevated in birds with metabolic stress induced by PHS and TD because both conditions could produce damage in tissues such as the lungs and growth plate cartilage, respectively. However, from the results, it appears that OVT is only elevated in the event of infection or tissue injury, not in diseases that may not involve inflammation. Accordingly, using femoral head separation disorder, a different metabolic

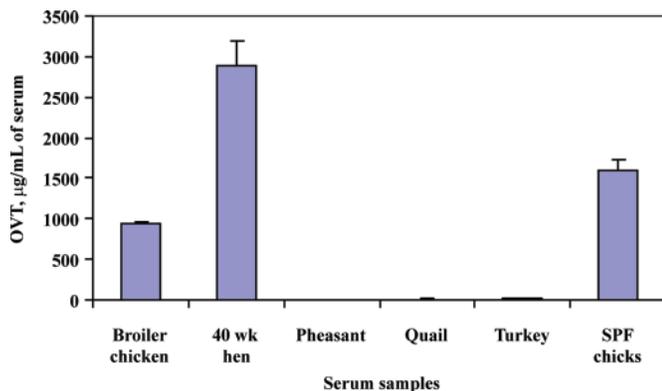


Figure 2. Ovotransferrin (OVT) concentrations of serum samples of chickens and other avian species measured by enzyme immunoassay ($n = 5$). SPF = specific-pathogen-free.

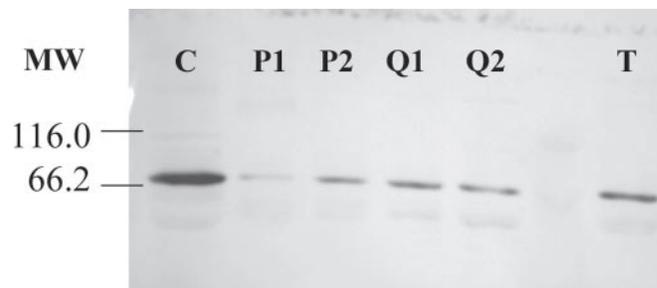


Figure 3. A Western blot result showing the recognition of ovotransferrin by the anti-chicken transferrin antibody. C = chicken; P = pheasant; Q = quail; T = turkey. MW = molecular weight.

Table 1. Serum ovotransferrin levels of chickens with different health conditions¹

| Disease type | Treatment or health conditions (age) ² | Control ³ (n) | Experimental ⁴ (n) |
|---------------------|---|--------------------------|-------------------------------|
| Metabolic | PHS/ascites (6 wk) | 1.65 ± 0.12 (n = 12) | 1.50 ± 0.20 (n = 18) |
| | Thiram TD (2 wk) | 0.93 ± 0.09 (n = 13) | 0.92 ± 0.06 (n = 13) |
| Infectious | Spontaneous TD (6 wk) | 1.19 ± 0.27 (n = 11) | 0.92 ± 0.18 (n = 11) |
| | <i>Escherichia coli</i> infection (3 wk) | 1.33 ± 0.10 (n = 10) | 2.44 ± 0.25* (n = 10) |
| | <i>Eimeria maxima</i> (2 wk) | 1.12 ± 0.18 (n = 10) | 2.00 ± 0.24* (n = 12) |
| | <i>Eimeria tenella</i> 5 d PI (27 d) ⁵ | 1.20 ± 0.16 (n = 7) | 4.88 ± 0.37* (n = 9) |
| Autoimmune vitiligo | <i>E. tenella</i> 10 d PI (32 d) ⁵ | — | 2.96 ± 0.32* (n = 6) |
| | Smyth line chickens (13 wk) | 0.94 ± 0.07 (n = 8) | 1.68 ± 0.13* (n = 7) |
| | Brown line chickens (13 wk) | 1.15 ± 0.08 (n = 8) | NA ⁶ |

¹The results are expressed as milligrams per milliliter of serum (mean ± SEM). The statistical results reflect comparisons within rows. Unless specified, all experiments were done using broiler chickens.

²PHS = pulmonary hypertension syndrome; TD = tibial dyschondroplasia; PI = postinfection.

³Control = nonaffected birds.

⁴Experimental = affected birds.

⁵Specific-pathogen-free chickens were used for *E. tenella* infection experiments.

⁶NA = not applicable.

*Indicates significantly different ($P \leq 0.05$) from their respective controls.

skeletal problem in poultry, we did not observe any change in their serum OVT concentrations (Durairaj et al., 2009). Ovotransferrin elevation was also evident from the evaluation of sera from Smyth line birds with active vitiligo, an autoimmune-autoinflammatory disease resulting in the loss of melanocytes, hence pigmentation, in feathers. Increased levels of proinflammatory cytokines such as the interleukins 1 and 6 and tumor necrosis factor α after infection or immunomodulation are implicated in the stimulation of APP production by the liver and increase their levels in blood (Tosi, 2005). Similar mechanisms may be applicable for the elevation of OVT levels in chickens. Ovotransferrin remains elevated as long as the inflammation persists (Xie et al., 2002b; Rath et al., 2008). An elevated level of OVT during infection may serve as an immunomodulator (Xie et al., 2003), prevent microbial growth (Giansanti et al., 2007), and possibly act as an antioxidant against Fenton reaction mechanisms, which potentially generate free radicals involved in oxidative tissue damage (Superti et al., 2007). Also, OVT has been shown to be associated with the angiogenesis process in chickens (Cermelli et al., 2000). Angiogenesis is an important aspect of postinflammatory wound healing (Eming et al., 2007; Velazquez, 2007), and the elevation of OVT may be a protective mechanism responsible for restoring homeostasis.

In conclusion, our results suggest that monitoring the serum level of transferrin can indicate the presence of inflammation in chickens regardless of its origin and therefore, can provide a diagnostic and prognostic marker for flock health and welfare.

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