ENZYME-LINKED IMMUNOSOBENT ASSAY TO MEASURE SERUM FERRITIN IN TOUCANS (*RAMPHASTIDAE* SP.)

by

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Abstract

Background: Iron storage disease has proven to be a serious health concern for captive toucans. Physiologic mechanisms to efficiently extract iron from naturally iron-deficient diets appear the likely cause of iron overload when fed iron-sufficient diets in captivity. Iron overload can result in diabetes, heart failure, and even death. Serum ferritin concentrations are considered the most reliable screening tool to predict total body iron stores in many species, but an assay has not been available to measure serum ferritin in toucans.

Objective: The purpose of this study was to develop an enzyme-linked immunosorbent assay (ELISA) to measure serum ferritin in toucans using a polyclonal antibody in a sandwich arrangement.

Methods: Ferritin was isolated from toucan liver and used as a standard. A rabbit polyclonal anti-toucan antibody was used as the capture antibody and as a detection antibody conjugated to horseradish peroxidase. Linearity of toucan ferritin standards, effect of serum dilution, recovery of added ferritin standards, and intra- and inter-assay variability were determined.

Results: Ferritin standards were linear from 0 to 50 ng/ml. The relationship between serum dilution and serum ferritin concentration was also linear. When 10, 20, 30, 40, or 50 ng/ml of purified toucan ferritin were added to diluted serum, the recoveries varied from 69% to 104%. The intra-assay variability for four test serum samples averaged 11% and the inter-assay variability for the same four samples averaged 11%.

Conclusions: Although the results from the linearity and recovery studies are promising for assay development when viewed independently, preliminary ferritin concentrations

from all toucans studied are much higher than expected. Upon further evaluation including Dot blot assays, Western blot assays, SDS-PAGE, and protein determination of the ferritin stock solution, it was determined that the ferritin stock solution did not contain a pure protein and therefore likely renders the assay invalid. Further testing is needed to confirm these findings.

Table of Contents

List of Figures

List of Tables

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Chapter 1 - Introduction

Ferritin

Structure and Function

Ferritin is a ubiquitous, water soluble, multimeric iron storage protein composed of an apoprotein shell containing 24 subunits that form a hollow sphere in which iron is stored (Fig.1.1).[1] The subunits consist of two distinct types with different molecular weight, immunoreactivity, and amino acid composition.[2] The H, or heavy, subunit is approximately 21 kDa and the L, or light, subunit is approximately 19 kDa.[2] Isotypes of ferritin containing different proportions of each subunit are found in different cells and organs throughout the body.[2] The configuration of the subunits results in the formation of channels through which iron is able to pass into and out of the molecule.[2] Iron is taken up in the ferrous form, but oxidized to the ferric form at the surface of the apoprotein shell and stored in the core of the molecule (Fig.1.1).[1,3] One ferritin molecule can store up to 4700 ferric iron ions.[1] Iron binding to ferritin is important not only for iron storage and availability for hematopoiesis, but to protect cells from the damaging free radicals that are produced from free iron.[4,5]

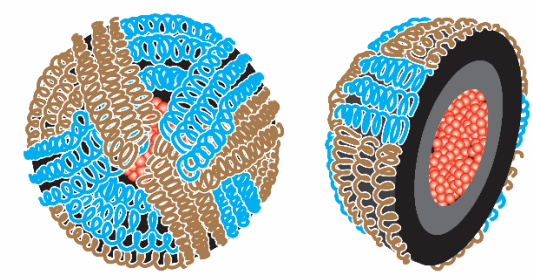


Figure 1.1- Ferritin structure. Heavy and light ferritin subunits (brown and blue) surround a core of ferric iron ions (red).

Synthesis and Storage

The liver hepatocytes synthesize ferritin within the polysomes and rough endoplasmic reticulum.[6,7] Although ferritin can be found in all cells of the body, the liver is the major iron storage organ in many species including humans, while cattle and horses tend to store more iron in the spleen.[8] For those species that store iron in the liver, the hepatocytes are the predominant iron storage cell followed by Kupffer cells.[2] These cells mobilize iron in times of need, such as erythropoiesis, by releasing iron to apotransferrin.[2] The rate of ferritin synthesis is dependent on the presence of iron and the iron status of the cell.[9-11]

Measurable forms

Although predominately intracellular, ferritin is present in both tissue and serum forms.[12] Tissue ferritin can be quantified by directly measuring nonheme iron concentrations in various organs, most often liver and spleen.[5] The origin of serum ferritin is unknown, but is likely derived from hepatocyte sources.[13] Serum ferritin, unlike tissue ferritin, has low iron content and is suspected to generally be actively secreted from cells rather than by cellular leakage.[14-17] Serum ferritin has a blood half-life of approximately 30 hours.[18-20] Tissue ferritin is cleared from the blood much faster with a half-life of 3-30 minutes, thus the majority of ferritin measured in blood is the serum form rather than the tissue form.[18,20-24] The prolonged half-life of serum ferritin appears to be related to the presence of carbohydrate on the molecule that is not present on the tissue form.[16,18]

Human Serum Ferritin Assays

An assay to measure serum ferritin in humans was first developed by Reissman and Dietrich in 1956.[12] Their method involved precipitation of ferritin with a rabbit antihuman ferritin antibody and measurement of the amount of iron in the precipitate. This assay was not sensitive enough to detect the minute concentrations of serum ferritin present in healthy patients. A more sensitive immunoradiometric assay was developed in 1972 that was able to detect serum ferritin in normal patients.[25] Two immunoradiometric assays (IRMA), one utilizing a polystyrene bead (bead IRMA) and the other a polystyrene tube (tube IRMA), and a radioimmunoassay (RIA) to measure serum ferritin in humans have been developed. [26,27] The bead IRMA has been found to have superior variability compared to the tube IRMA and better precision than the RIA. When evaluating patients with serum ferritin within the healthy reference interval, the RIA and IRMA assays performed similarly.[27] Currently there is also a sandwich ELISA kit available to measure serum ferritin in humans (Eagle Biosciences, Inc., Nashua, NH).

Animal Serum Ferritin Assays

Numerous assays have been developed to measure serum ferritin in animal species. A sandwich ELISA to measure serum ferritin in pigs was developed in 1983.[28] A polyclonal antibody was used to react with porcine ferritin purified by gel filtration. Attempts to react the porcine ferritin with both antihuman and antiequine antibodies were unsuccessful. The antiporcine antibody also did not react with human or equine ferritin proving that ferritin appears to be generally species specific and subsequent assays would need to be developed for each species of interest. The primary use of this assay is

intended to evaluate piglets for iron deficiency as often occurs during growth if not supplemented.

An ELISA to measure serum ferritin in horses has been developed primarily to monitor iatrogenic iron overload in horses given iron supplements to improve performance, but also is valuable for identifying iron deficiency.[29] This assay uses commercially available equine ferritin standards and antiequine antibody. The equine serum ferritin assay has also been adapted to measure ferritin in rhinoceros sera.[30] Although ferritin is generally species specific, cross-reactivity was observed between the antiequine antibody and rhinoceros ferritin. Black rhinoceroses are susceptible to acute hemolytic anemia as well as hemosiderosis when kept in captivity; the ability to measure serum ferritin is helpful when monitoring for both diseases.[30]

Two sandwich ELISA assays have been developed to monitor iron stores in dogs. The canine ferritin for both was purified by a gel filtration technique.[31,32] The first assay used a monoclonal antibody as both the capture and detector antibody.[31] This assay was later replaced by an assay that uses separate murine anticanine monoclonal antibodies for capture and detection.[32] The use of separate capture and detector antibodies increased specificity for ferritin resulting in improved coefficients of variation and the assay does not require serum dilution due to a broader standard curve.[32] Cross-reactivity between the anticanine antibody and fur seal ferritin allowed use of the canine assay to measure serum ferritin in fur seals which are susceptible to iron storage disease in captivity.[33]

Captive lemurs also accumulate iron in organs resulting in disease and death.[34] A sandwich ELISA to measure serum ferritin in lemurs utilizes cross reactivity between a rabbit polyclonal antihuman antibody and lemur ferritin.[35]

A feline serum ferritin assay similar to the canine assay has also been developed and is used to evaluate for iron deficiency.[36] That assay requires the use of 2-mercaptoethanol in the dilution solution to reduce disulfide bonds on the ferritin and allow binding to the antibody. In 1982, serum ferritin concentrations were measured in dairy cows using an immunoradiometric assay to identify iron deficiency during pregnancy and lactation.[37]

Avian Serum Ferritin Assays

At this time, only two assays have been developed to measure serum ferritin in avian species. A radioimmunoassay reported to measure duck ferritin.[38] Dilutions of 1:50 were used to obtain concentrations within the standard curve range. According to the authors this dilution was necessary to bring the high duck serum ferritin concentrations within the standard curve range. A radioimmunoassay to measure serum ferritin in hens is also reported and demonstrated higher serum ferritin concentrations in chickens than humans[39]. In this study the authors used serum dilutions of 1:16 to 1:64 and reported a working range of the assay to be 21.8 ng/mL to 175 ng/mL.

Correlation of Serum Ferritin with Tissue Iron Stores

Many of the assays developed for animals have been used to evaluate the correlation of serum ferritin concentration with tissue iron stores. Serum ferritin has proved to correlate with tissue nonheme iron stores in humans, dogs, pigs, horses, and cats.[29,32,36,40-42] Serum ferritin in dogs, cats, and horses was shown to better predict iron stores than serum iron or serum total iron binding capacity.[29,36,41] In pigs, both serum

ferritin and serum total iron binding capacity correlated with liver and spleen iron stores while serum iron, erythrocyte count, hemoglobin, packed cell volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration were not good estimates of tissue iron stores.[42]

Serum Ferritin Aberrations in Humans

In humans, serum ferritin values have been shown to fluctuate based on physiologic variations. Throughout infancy ferritin concentrations decrease as tissues stores are mobilized to be used in hematopoiesis.[43] Once growth is complete in early adulthood of men, ferritin values increase.[44] An increase is not generally seen in women until after menopause.[45] Pregnant women often experience a decrease in ferritin beyond the first trimester unless iron supplements are taken.[46]

Pathologic states that increase serum ferritin include liver or other ferritin-rich tissue damage, inflammation and infection, hereditary hemochromatosis, rheumatoid arthritis, and malignancy.[6,14,47-49] Types of neoplasia that often result in increased ferritin concentrations in humans include pancreatic carcinoma, lung cancer, hepatoma, nephroblastoma, metastatic breast cancer, acute leukemia, and Hodgkin's disease.[47] Proposed reasons for the increased ferritin with neoplasia include increased storage iron due to inflammation, liver damage, tissue necrosis, or direct release from the tumor.[17,47,50,51] Other influences on ferritin concentrations in humans include heavy exercise and inflammation.[43,52]

Decreased serum ferritin concentrations in humans have been documented with iron deficiency, hypothyroidism, and ascorbate deficiency.[17] Serial serum ferritin

measurements are often used in humans as a noninvasive way to monitor conditions in which iron deficiency anemia is expected to develop, such as in patients undergoing repeated phlebotomy, gastric surgery leading to malabsorption of iron, and women with increased menstrual blood loss.[17]

Serum Ferritin Aberrations in Animals

Fewer studies have been done in animals, but similar findings are expected. Similar to humans, serum ferritin has been shown to be increased in horses after moderate to heavy exercise.[53] Conditions which result in increased storage iron, such as chronic hemolytic anemia and hemochromatosis secondary to repeated blood transfusions also are expected to result in increased serum ferritin.[54-56] Several neoplasms including malignant histiocytosis, lymphoma, splenic hemangiosarcoma and hemangioma have all been shown to result in increased serum ferritin in dogs.[55,57-59] Ferritin is an acute phase protein and is expected to be increased in inflammatory conditions due to TNF-α-stimulated ferritin secretion from hepatocytes.[60-62]

Iron storage disease is a major cause of increased serum ferritin in captive fur seals, lemurs, and rhinoceroses.[30,33,35] Iron storage disease is also common in many captive avian species including toucans, mynahs, birds-of-paradise and starlings, but a correlated increase in serum ferritin has not yet been confirmed due to lack of available assays.[63]

Decreased serum ferritin values are observed in animals with iron deficiency.[61,64,65] Serum ferritin measurement is particularly useful in differentiating true iron deficiency anemia from anemia of inflammation. In both conditions, serum iron will be

low but serum ferritin is expected to be increased with inflammation as it has been proven to be an acute phase protein.[2,5,17,47] However, ferritin's role as an acute phase protein may complicate diagnosis of iron storage disease when concurrent inflammatory conditions are present. The ability to measure serum ferritin to diagnose and monitor these conditions is particularly useful in animal species where more invasive methods of determining iron stores are often risky, expensive, and cost prohibitive.

Chapter 2 - Hypothesis and Objectives

Hypothesis

1. Serum ferritin can be measured in toucans using an enzyme-linked immunosorbent assay with a polyclonal rabbit antitoucan ferritin antibody.

Objectives

1. Develop an enzyme-linked immunosorbent assay to measure serum ferritin in toucans (*Ramphastidae sp*.)
2. Extend the use of the toucan serum ferritin assay to other avian species.

Chapter 3 - Enzyme-linked immunosorbent assay to measure serum ferritin in toucans (*Ramphastidae* sp.)

Introduction

Iron storage disease (ISD), defined as iron overload in tissues resulting in organ dysfunction, is a serious concern in many captive avian species.[63,66-73] Frugivorous and insectivorous species including Ramphastidae (toucans), Paradisaeidiae (birds-of-paradise), Sturnidae (mynahs, starlings), Thraupidae (tanagers), and Bucerotidae (hornbills) are more susceptible to ISD than their granivorous or omnivorous counterparts.[63,66-75] Genetic and evolutionary theories on the cause of ISD susceptibility in these species have been proposed with strong evidence supporting physiologic adaptations that accommodate for the scarcity of dietary iron in the wild with very efficient dietary iron extraction.[63,66-71] When fed diets with higher iron content in captivity, this efficient intestinal iron absorption results in iron accumulation in the form of hemosiderin and ferritin in tissues, including the liver, spleen and heart, and may increase the risk of hepatic cirrhosis, diabetes, hepatic neoplasia, heart failure and microbial infections.[63,66,68-71,74]

Clinical signs of iron storage disease include weight loss, depression, ascites, dyspnea, feather picking, vestibular disturbances, and death.[63,70,73,75] Serum biochemical data is nonspecific but may include hypoalbuminemia and increased hepatic enzymes.[68] Serum iron, total iron binding capacity, and percentage iron saturation are nonspecific but may yield support for a diagnosis of ISD, however liver histopathology with iron quantification is considered the only definitive test.[68] Collection of liver tissue is expensive and invasive making it impractical for repeated monitoring of ISD. Serum

ferritin concentrations have been used as a reliable screening tool for tissue iron stores in many species, but while immunologic cross-reactivity between ferritins from some species has been observed, ferritin is generally species-specific and until now an assay has not been commercially available for birds.[29,30,33,35,37,41,42,76,77] Here we describe the development of an ELISA to measure serum ferritin in toucans using a polyclonal rabbit antitoucan ferritin antibody.

Materials and Methods

Ferritin was isolated from five Toco toucan (*Ramphastos toco*) liver samples by previously described methods.[31,78] All five toucans were all diagnosed with iron storage disease by histologic examination of the tissues at the time of necropsy. The purity of the ferritin was confirmed using SDS-PAGE chromatography and compared to equine ferritin standards. Rabbit polyclonal antibodies to toucan ferritin purified by protein G affinity chromatography (QED Bioscience, Inc., San Diego, CA) were used as the first (capture) antibody and as a detector antibody conjugated to horseradish peroxidase.

ELISA Solutions

Sodium carbonate buffer (10 mM pH 9.6) was used to coat the test plates with the capture antibody. Phosphate buffered (0.02 M) saline (0.15 M) (PBS) containing 0.1% polyoxyethylene sorbitan monoleate (Tween 80, Sigma Chemical Co., St. Louis, MO) and 0.5% bovine serum albumin was used as a blocking buffer to fill unoccupied protein-binding sites on the 96-well plates, and to dilute the sera and the toucan ferritin stock. The plates were washed with PBS containing 0.1% Tween 80 (wash solution). Ferritin standards of 10, 20, 30, 40, and 50ng/mL were prepared using purified toucan ferritin stock diluted in blocking buffer. Premix ABTS microwell peroxidase substrate containing 2,2'-azino-di (3-ethyl-benzthiazoline-6-sulfonate) as a chromogen (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used for color development.

ELISA Procedure

Flat-bottom polystyrene 96-well EIA/RIA high-binding microtitration plates (Costar®; Corning Inc., Corning, NY) were used as test plates, and 100 µL of polyclonal antitoucan ferritin diluted to 18 µg/mL in 10 mM sodium carbonate buffer pH 9.6 were

added to each well. The plates were sealed with sealing tape and incubated at 37^0C for 2 hours. The plates were then stored at -20^0C until use. Frozen plates were thawed at 37^0C for 30 minutes prior to use. The plates were washed five times with wash solution. 200 µL blocking solution were added to the wells and the plates were then incubated at 37^0C for 30 minutes. The plates were washed five times with wash solution and tapped dry before diluted sera, standards or test sera were added to individual wells (100 µL/ well). The plates were then incubated at 37^0C for 2 hours. After washing the plates five times with wash solution, 100 µL of horseradish peroxidase labeled polyclonal antitoucan ferritin antibody diluted to 4.5 µg/mL in dilution solution were added to each well. The plates were incubated for 2 hours at 37^0C. After washing five times with wash solution, 100 µL of ELISA peroxidase substrate solution were added to each well and the kinetic absorbance change from 410 nm was recorded in an automated 96-well plate reader (VersaMax; Molecular Devices, Sunnyvale, CA). Computer software (SoftMax Pro 5.4, MDS Analytical Technologies, Sunnyvale, CA) was used to calculate the serum ferritin concentration from the recorded kinetic absorbance as it relates to known ferritin standards using a linear standard curve. The reported ferritin concentration was multiplied by the dilution factor to determine the actual serum ferritin concentration of the sample.

Data Analysis

Generalized linear mixed models (GLMM) were used to evaluate the association between ferritin standards with test serum ferritin concentration levels. Generalized linear mixed models (GLMMs) provide a useful framework for analyzing normal and non-normal data, by using link functions and exponential family distributions (e.g., Gaussian,

binomial), while accounting for the design and hierarchical structure of the study through the incorporation of random effects (e.g., samples nested within plates).[79] The outcome of the model was the concentration of serum ferritin in ng/mL, which was recorded and modeled on a continuous scale. In all evaluations, the mean of three wells was used as the ferritin concentration for a particular sample. Our exposure variable, ferritin standards, was modeled as a categorical variable (ferritin standard concentrations of 0, 10, 20, 30, 40 and 50 ng/mL). Models were run using Proc Glimmix (SAS 9.3, SAS Institute Inc., Cary, NC), with a Gaussian distribution, identity link, restricted pseudo-likelihood estimation, Kendall-Rogers degrees of freedom and random effects for ferritin standards clustered within assay plates. Model-adjusted least square means and 95% confidence intervals for concentrations of serum ferritin in 0 to 50 ng/mL standards are presented in Table 1. *P*-values lower or equal than 0.05 were considered statistically significant. Figure 1 depicts the model-adjusted association between standard concentrations and observed levels of serum ferritin in ng/mL.

Assay Evaluation

Effect of serum dilution on serum ferritin concentrations

GLMMs were used to model the association between serum dilution (100,000x; 200,000x; 400,000x and 800,000x) of toucan serum samples (n = 38) with serum ferritin concentration levels. The outcome of the model was the concentration of serum ferritin in ng/mL and the exposure variable consisted of 100,000x; 200,000x; 400,000x and 800,000x dilutions of toucan serum samples. Characteristics of the model were specified above. Model-adjusted least square means and 95% confidence intervals for concentration of serum ferritin in diluted sera are presented in Table 3.2, Fig 3.2.

Linearity of toucan ferritin standards

Linearity for ferritin standard concentrations added to diluted sera, was determined by using similar GLMMs as described above. The outcome of the model was the concentration of serum ferritin in ng/mL and the exposure variable consisted of 100,000x serum dilutions with 0 to 50 ng/mL ferritin standard concentrations added. Model-adjusted least square means and 95% confidence intervals for concentration of serum ferritin in diluted sera with 0 to 50 ng/mL standards added are presented in Table 3.3.

Percent recovery of ferritin standard added to toucan serum samples

The percent recovery of ferritin from serum was calculated using the ferritin concentration in diluted sera containing added ferritin and the same sera without the added ferritin (Table 3.4). The observed ferritin concentration was divided by the expected ferritin concentration for a given concentration of added ferritin.

Intra-assay and inter-assay variation

Using a 100,000x dilution, four test sera (IDs 102, 105, 107 and 109) were tested on 10 to 11 different plates to monitor plate-to-plate variation on serum ferritin levels. The serum samples were aliquoted and kept frozen at -20˚C between assays. The overall mean serum ferritin concentration, the standard deviation and the coefficient of variation (CV = standard deviation/mean) were calculated for each of the four test sera and across all controls (Inter-assay CV) on different days over a period of one month (Table 3.5). Intra-assay CVs, calculated by determining the ferritin concentration in each test sample run 6-8 times on the same plate were provided by the plate reader (VersaMax; Molecular Devices, Sunnyvale, CA) (Table 3.5).

Dot Blot Solutions

The wash solution consisted of 1X PBS with 0.05% Tween-20 (PBS-T). The blocking solution (NFDM) was prepared from 5% (w/v) non-fat dry milk powder (Great Value, Wal-Mart Inc., Bentonville, AR) in 1X PBS with 1% Tween-20 and was used to block any unused binding sites.

Dot Blot Procedure

A dot blot assay was performed to confirm specific binding of the antitoucan antibody binding to the toucan ferritin stock solution. Toucan ferritin standard, canine ferritin standard, and the ELISA blocking solution were diluted in phosphate buffered saline at serial dilutions of 1:10, 1:20, 1:40, 1:80, and 1:160. Ten microliters of each dilution were applied to Polyvinylidene difluoride (PVDF) membranes (Cat# RPN303F, Amersham, GE Healthcare Life Sciences, Pittsburgh, PA). The membranes were allowed to dry then blocked with NFDM for one hour at room temperature. After three washes of 5 minutes each with PBS-T, each membrane was incubated for 1 h at room temperature with horseradish peroxidase conjugated anti-toucan ferritin antibody. The conjugated antibody was diluted 1:100, 1:500, 1:1000, or 1:2000 in NFDM. After a washing step, the antibody reactivity was detected by incubating the membranes with 4-chloro-1-naphtol/3,3'-diaminobenzidine, tetrahydrochloride (CN/DAB) substrate (Cat # 34000, Thermo Fisher Scientific Inc., Waltham, MA) for 5-10 minutes or until spots were visible. The reactions were stopped by rinsing the membranes in double distilled water. Membrane pictures were acquired using UVP GelDoc-It Imaging System (UVP, LLC, Upland, CA).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Solutions

The 4X sample buffer consisted of 8% SDS, 40% glycerol, 25 mM Tris-HCl, 20% β-mercaptoethanol, and bromophenol blue.

SDS-PAGE Procedure

An SDS-PAGE was performed to confirm the ferritin stock solution contained only pure ferritin protein and to rule out degradation of the protein. Sodium Dodecyl Sulfate-Polyacrylamide gel according to the method described by Laemli (1970) was used to separate the protein samples. A 1 mm thick 12% resolving gel and a 5% stacking gel, were casted using Biorad vertical gel apparatus system (Biorad, Hercules, CA). Ferritin standards and sera samples were mixed with 4X sample buffer and boiled for 5 minutes. Samples were then briefly centrifuged and loaded into the gel. The electrophoresis was performed at 150 V for 45 minutes. The gel was then stained using Simply Blue Safe Stain (Life Technologies, Grand Island, NY). Briefly, the gel was washed three times (one minute/each) with boiled double distilled water; the gel was then stained for 5 minutes with heated Simply Blue Safe Stain and distained in double distilled water for 10 minutes to overnight. A gel picture was obtained using UVP GelDoc-It Imaging System (UVP, LLC, Upland, CA).

Western Blot Solutions

The wash solution was prepared using 1X PBS with 0.05% Tween-20 (PBS-T). A blocking solution (NFDM) consisting of 5% (w/v) non-fat dry milk powder (Great Value, Wal-Mart Inc., Bentonville, AR) in 1X PBS with 1% Tween-20 was used to block any unused binding sites.

Western Blot Procedure

Western Blot was used to identify nonspecific antibody binding to other proteins in toucan serum. The protein samples separated by 12% SDS-PAGE were blotted into a PVDF membrane using the Mini Trans-Blot Electrophoretic Transfer Cell (Biorad, Hercules, CA) following the manufacturer's instructions.

The protein transfer was obtained by applying a current of 100 V for 1 hour. The membrane was blocked overnight at +4°C with NFDM. The blocking step was followed by three washes with PBS-T.

Anti-toucan antibody conjugated to horseradish peroxidase diluted in NFDM was applied to the membranes and incubated for one hour at room temperature in shaking. After three washes in PBS-T, the antibody reactivity was detected by incubating the membranes with CN/DAB substrate (Cat # 34000, Thermo Fisher Scientific Inc., Waltham, MA) for 5-10 minutes or until bands were visible. The reactions were stopped by rinsing the membranes in double distilled water. A picture was acquired using UVP GelDoc-It Imaging System (UVP, LLC, Upland, CA).

Protein Concentration Determination

Protein concentration of the ferritin stock solution was performed to confirm the originally determined concentration was accurate. Two different kits were used to determine the protein concentration of the toucan ferritin stock solution, the Biorad Protein Assay Dye Reagent Concentrate and the Pierce BCA Protein Assay Kit. The manufacturer's instructions were followed for each kit and the values were determined based on the standard curve concentrations.

Results

Ferritin standards from 0 to 50 ng/mL were linearly associated with test serum ferritin concentrations ($P < 0.001$) (Fig. 3.1, Table 3.1). Serum dilutions of 100,000x; 200,000x; 400,000x; and 800,000x were significantly ($P < 0.001$) linearly associated with serum ferritin concentrations. Adjusted least square mean ferritin concentrations for the 100,000x; 200,000x; 400,000x; and 800,000x dilutions were 88.82 ng/mL, 63.97 ng/mL, 39.52 ng/mL, and 14.81ng/mL, respectively (Fig. 3.2, Table 3.2). When purified ferritin standards were added to three different toucan serum samples diluted by a factor of 100,000, recovery of the purified ferritin standard varied from 89.04% to 104.25% when 10 ng/mL standard was added, 78.86% to 93.10% when 20 ng/mL standard was added, 71.13% to 88.62% when 30 ng/mL standard was added, 69.30% to 95.50% when 40 ng/mL standard was added and was 91.51% when 50 ng/mL standard was added (Table 3.3, Table 3.4). The intra-assay variability for four sera averaged 11% whereas the inter-assay variability for the same four samples averaged 11% (Table 3.5).

The dot blot assay demonstrated evidence of antitoucan antibody binding to the toucan ferritin at all antibody dilutions with optimum sensitivity at the 1:500 antibody dilution and no binding was observed to either the canine ferritin or the blocking buffer containing bovine serum albumin (Fig.3.3). The western blot assays did not reveal antibody binding to any of the proteins or sera tested at any of the antibody dilutions. Determination of the protein concentration of the ferritin stock solution was performed three times. The Biorad kit had resultant values of 1.4 mg/mL and 2.5 mg/mL. The Pierce BCA kit yielded a value of 3.3 mg/mL. SDS-PAGE revealed a thick protein band near the 19 kDA region and numerous other distinct bands of larger molecular weight (Fig. 3.4).

Discussion

We developed an ELISA to measure serum ferritin concentrations in toucans using rabbit polyclonal antibodies against toucan ferritin. Toucan ferritin standards were linear from 0 to 50 ng/mL (Fig.3.1, Table 3.1). Measured ferritin concentrations did not increase as expected with standard concentrations greater than 50 ng/mL, indicating inhibition of the assay at ferritin concentrations above 50 ng/mL (data not shown). Ferritin concentrations could not be determined in undiluted serum since the concentrations were greater than 50 ng/mL. Serum samples should be diluted by a factor of at least 100,000 before the assay is performed. We found that a 100,000 dilution was often sufficient to yield concentrations under 50 ng/mL; however greater dilutions are likely to be required for some individuals. Diluted samples with ferritin concentrations greater than 50 ng/mL should be further diluted and reassayed. The relationship between serum dilution and serum ferritin concentration was linear as observed by an expected decrease in measured ferritin concentration with increased serum dilution (Fig. 3.2). We attempted to validate this assay using serum from other avian species, including gulls, cormorants, birds-of-paradise, and swans, at similar dilutions; however we were unable to demonstrate an effect of sample dilution in those species using the toucan antibody (data not shown). We suspect this is due to antigenic dissimilarity of ferritin between species or the presence of a naturally occurring interferent in the serum of those species therefore; additional avian assays may require ferritin isolation from each individual species. When 10, 20, 30, 40, or 50 ng/mL of purified toucan ferritin standard was added to toucan serum diluted by a factor of 100,000, recoveries varied from 69-104% indicating fair to good analytical accuracy of the assay and a lack of strong interfering substances in the diluent or serum (Table 3.4). The wide confidence intervals reflect the small sample size (Table 3.3). The

intra-assay variability averaged 11% when four sera samples were measured 6-8 times on the same plate (Table 3.5). The inter-assay variability averaged 11% when 4 sera samples were measured on 10 or 11 plates over the period of one month (Table 3.5). These measures of variability are comparable to other ferritin assays and are indicative of fair to good assay reproducibility as well as of the stability of frozen toucan ferritin over the period of at least one month.[31-33,35,36]

While our data appear promising for the development of the assay, the actual calculated ferritin concentrations obtained are very high compared to mammalian standards and are likely incompatible with life. Given the large degree of dilution needed to get values under the standard curve, the calculated concentrations were often 1 g/dL or higher and were commonly 10-20% or more of the total protein concentration (data not shown). We do not have any historical or clinical information on the birds used in the assay development so we are unable to confirm any circumstances, such as breeding or molting, which may lead to increased serum ferritin concentration. The dot blot assay confirmed that the antibody did indeed bind to the ferritin protein (Fig. 3.3). To evaluate for nonspecific binding of the antibody to other proteins in toucan serum or the blocking buffer we performed numerous western blot assays using various dilutions of the test samples as well as the antibody. Unfortunately we were not able to demonstrate binding of the antibody to either of the toucan ferritin stock solution or any proteins in toucan sera or the blocking buffer. We suspect this may be due to antibody specificity for a conformational epitope on the ferritin that is lost when the protein is denatured in the gel, although it is uncommon for a polyclonal antibody to bind to only one epitope. So unfortunately we are unable to prove or disprove nonspecific binding of the antibody to

other proteins in toucan sera or the blocking buffer. A western blot performed using a nondenaturing gel or an immunoprecipitation assay may help prove our suspicion, but have not been performed at this time. The protein determination kits were used to confirm that the concentration originally measured of the ferritin stock solution was indeed correct as a falsely increased protein concentration would result in an erroneous standard curve and drive our calculated concentrations up. Two of the three runs using the test kits yielded values slightly higher than the original measured concentration while one was lower (data not shown). The value that was lower than the original measured concentration had a lower R^2 value compared to the others and, therefore, may be more inaccurate. It is unknown which value is most accurate or why there was so much variation between the runs and the kits, but it is unlikely that the concentration used in the assay development was falsely elevated enough to result in falsely increased calculated serum ferritin concentrations. The SDS-PAGE was performed to look for protein degradation of the ferritin stock solution, which would again affect the standard curve and falsely increase the calculated serum ferritin concentrations. It was also performed to confirm purity of the sample. No evidence of protein degradation was observed, however the fact that there were numerous other distinct protein bands proved that the sample is not a pure protein (Fig. 3.4). Although the predominant protein band is approximately 19 kDa which likely correlates with the ferritin subunits, the other protein bands present pose an interesting dilemma. While it is not uncommon to have some impurities with a protein isolated and purified using a gel filtration technique, it is very possible that the antibody that was produced using the impure sample is binding to other proteins in the birds' serum.

At this time, we cannot be sure of the validity of our assay. It is likely that ferritin isolation and purification may need to be repeated, possibly by methods other than gel filtration. While serum ferritin concentrations have been shown to correlate with tissue iron concentrations in many species including cats, dogs, horses, cattle, pigs, and humans, further studies are required to evaluate the degree of correlation between serum ferritin concentration and tissue iron stores in toucans.[29,36,37,41,42,76] Until the degree of correlation is determined, the analytical sensitivity and specificity of this assay cannot be evaluated. At this time a valid reference interval for toucan serum ferritin has not been established; therefore this assay may prove more useful when used to monitor ferritin concentrations rather than to diagnose iron storage disorders. As with tissue iron storage, serum ferritin concentrations may be affected by other conditions such as hemolytic or inflammatory disease, which will affect the specificity of the assay for detecting iron storage disease.[5] This assay may help facilitate non-invasive monitoring of ferritin concentrations in captive toucans at risk for developing iron storage disease or toucans previously diagnosed with iron storage disease that are undergoing dietary modification or chelation therapy. Further studies are needed to evaluate the antibody specificity of this assay before the usefulness of the assay can be confirmed.

Table 3.1- Model-adjusted linearity of toucan ferritin standards.

Standard concentration (ng/mL)	n	Mean (ng/mL)	Mean 95% CI (ng/mL)
0	11	2.99	-0.47-6.46
10	9	12.32	8.49-16.15
20	10	18.97	15.33-22.60
30	7	26.21	21.87-30.56
40	6	33.95	29.26-38.65
50	2	45.04	36.90-53.18

CI = confidence interval

Mean = least square mean

Table 3.2 - Model –adjusted least square mean and standard errors of serum ferritin concentrations and serum dilution of toucan samples (n=38).

Dilution	Mean (ng/mL)	SEM (ng/mL)
100,000	88.82	3.91
200,000	63.97	4.36
400,000	39.52	4.48
800,000	14.81	5.38

SEM = standard error of the mean

Mean = least square mean

Table 3.3 - Model-adjusted least square mean and 95% CI of serum ferritin concentration in diluted sera with 0 to 50 ng/mL standards added.

Standard concentration added (ng/mL)	n	Mean (ng/mL)	Mean 95% CI (ng/mL)
0	3	33.71	8.13-59.29
10	3	40.55	14.98-66.13
20	3	44.60	19.02-70.17
30	3	50.22	24.65-75.80
40	3	61.29	35.72-86.87
50	1	76.08	56.12-99.05

CI=confidence interval

Mean = least square mean

Table 3.4 - Percent recovery of ferritin standard added to toucan serum samples (100,000x dilution).

Control ID	102		105		109	
Standard Added (ng/mL)	Observed (ng/mL)	Recovery (%)	Observed (ng/mL)	Recovery (%)	Observed (ng/mL)	Recovery (%)
0	38.41		45.37		17.35	
10	43.11	89.04	50.03	90.36	28.52	104.25
20	47.47	81.26	51.55	78.86	34.78	93.10
30	50.18	73.35	66.80	88.62	33.69	71.13
40	54.34	69.30	74.76	87.57	54.77	95.50
50	ND		87.28	91.51	ND	

ND= Not determined

Table 3.5 - Inter-assay and intra-assay coefficient of variation for four toucan sera samples.

	Inter-assay				Intra-assay			
Control ID	n	Mean (ng/mL)	SD	CV %	n	Mean (ng/mL)	SD	CV %
102	11	28.83	3.45	12	6	30.59	2.87	9
105	11	45.42	5.34	12	8	46.64	5.33	11
107	11	29.90	3.90	13	8	29.66	2.44	8
109	10	17.90	1.53	9	7	16.72	2.42	15
Mean CV				11				11

SD = standard deviation; CV = coefficient of variation

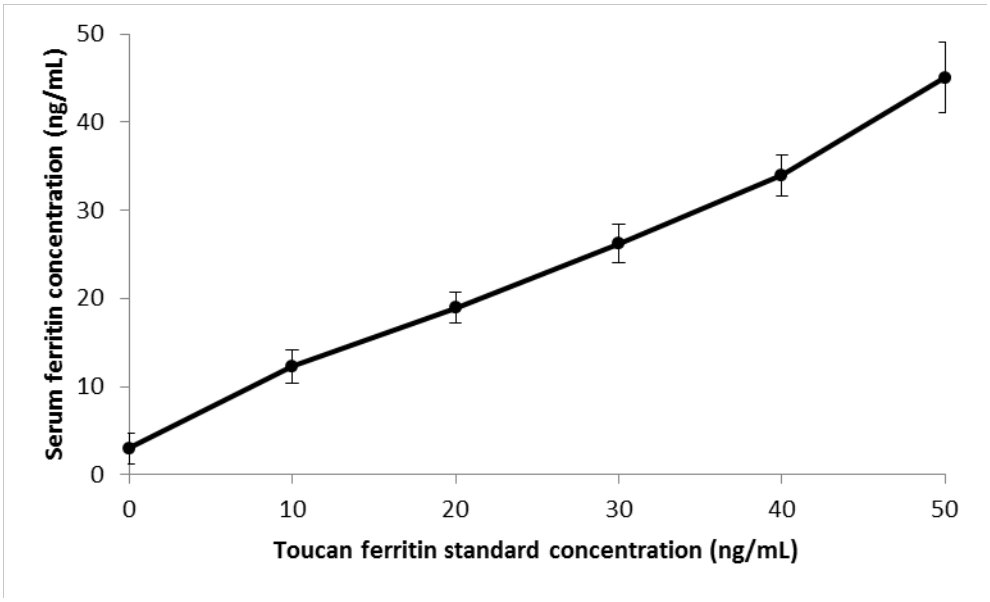


Figure 3.1 - Linearity of toucan ferritin standards.
Toucan ferritin standards were linear from 0 ng/mL to 50 ng/mL. Error bars represent standard error of the means.

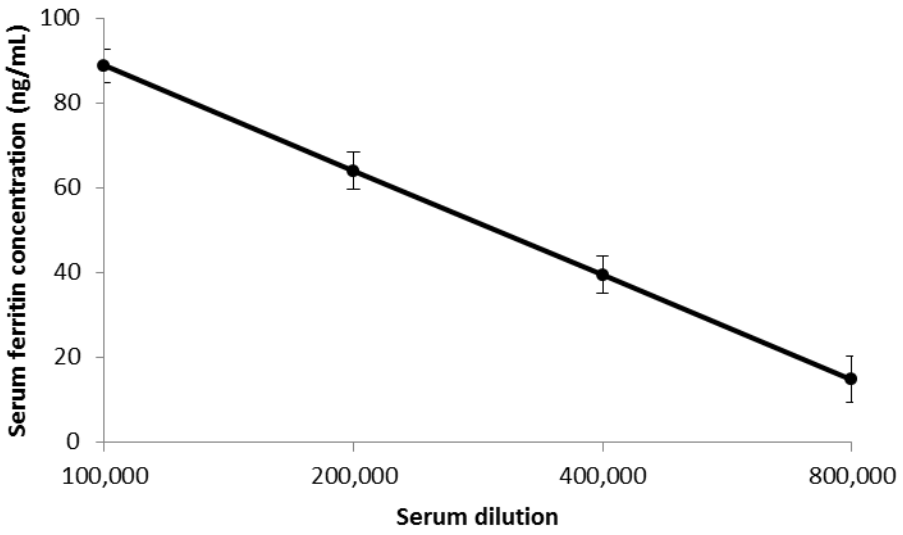
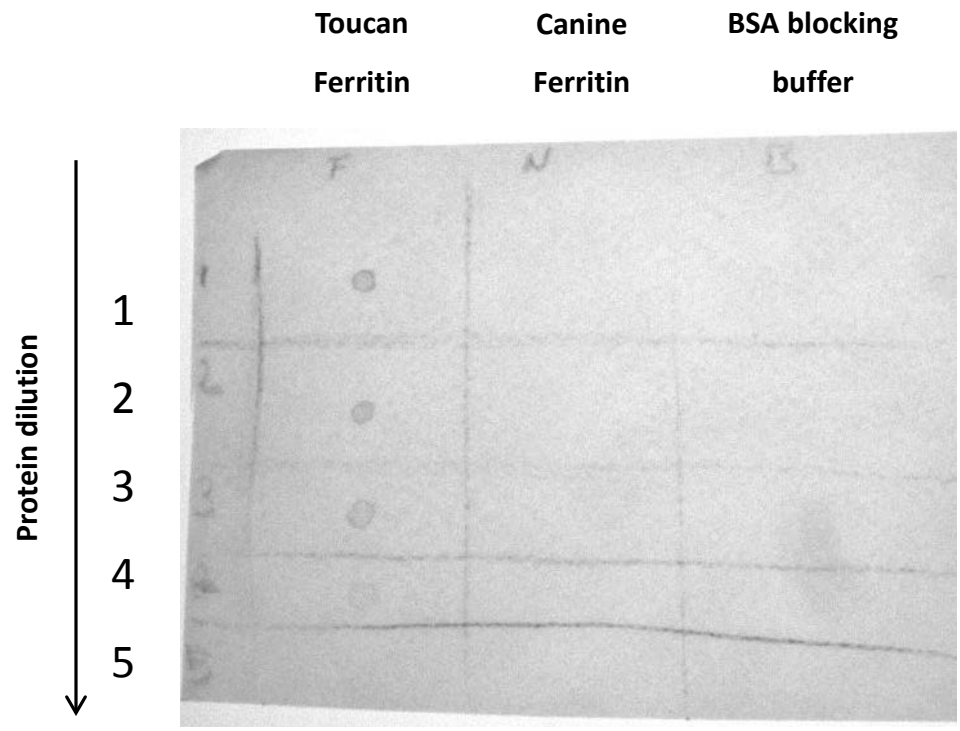


Figure 3.2 - Effect of serum dilution on serum ferritin concentration in toucans. Serum ferritin concentrations decreased proportionally with increased serum dilution.

Error bars represent standard error of the means.



Toucan Ferritin Dilution
1= Protein Stock diluted 1:10 in PBS
2= Protein Stock diluted 1:20 in PBS
3= Protein Stock diluted 1:40 in PBS
4= Protein Stock diluted 1:80 in PBS
5= Protein Stock diluted 1:160 in PBS

Canine Ferritin Dilution
1= Protein Stock Undiluted
2= Protein Stock diluted 1:10 in PBS
3= Protein Stock diluted 1:20 in PBS
4= Protein Stock diluted 1:40 in PBS
5= Protein Stock diluted 1:80 in PBS

BSA Blocking Buffer
1= BSA Buffer Undiluted
2= BSA Buffer diluted 1:10 in PBS
3= BSA Buffer diluted 1:20 in PBS
4= BSA Buffer diluted 1:40 in PBS
5= BSA Buffer diluted 1:80 in PBS

Figure 3.3 - Specific binding of the antitoucan antibody (1:500 dilution) to toucan ferritin was detected by dot blot assay.

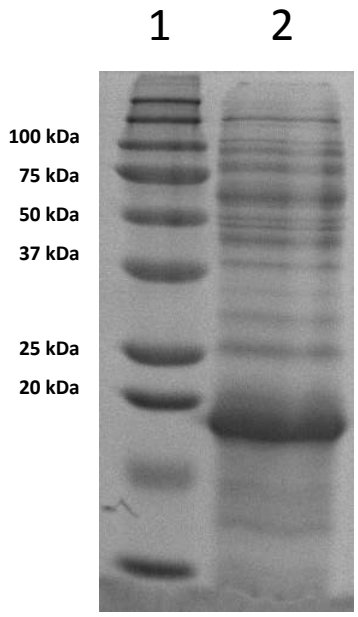


Figure 3.4 - SDS-PAGE containing a molecular weight marker (column 1) and undiluted toucan ferritin stock solution, 18 mg/well (column 2). A prominent band approximately 19 kDa (likely ferritin subunits) and many additional bands of higher molecular weight are observed in column 2.

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