Development of a sandwich enzyme-linked immunosorbent assay to detect and measure serum levels of canine ferritin.

RUNNING HEAD: SANDWICH ELISA FOR CANINE SERUM FERRITIN

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We established a homologous sandwich enzyme-linked immunosorbent assay (ELISA) to measure serum levels of canine ferritin. Our assay uses a rabbit anti-canine heart ferritin polyclonal antibody, and canine heart ferritin as a standard. Serum ferritin concentration in healthy dogs ($n = 163$) was $789 \pm 284$ ng/ml (mean ± standard deviation), a value higher than reported previously. Confidence levels relating to repeatability, dilution, and recovery for this method were high. Therefore, we believe our developed sandwich ELISA will be effective in evaluating serum levels of canine ferritin.

KEY WORDS: dog, ferritin, sandwich ELISA
Ferritin is a ubiquitous iron storage protein containing an iron core within a 24-mer globular protein. It consists of heavy (H) and light (L) subunits, with molecular masses of 21 and 19 kDa, respectively [4, 15]. Ferritin can accommodate 3,000–4,500 iron atoms, and protect cells from reactive oxygen species [5]. All cells in various mammalian species contain ferritin, with these cells mainly present in the liver, spleen and bone marrow [6]. In dogs, ferritin circulates in the serum at a relatively low concentration (< 1 μg/ml), and serum ferritin concentration is positively correlated with storage of iron in the body [12]. Serum ferritin levels are altered in certain disease states such as iron overload, inflammatory diseases, liver damage and malignancies [15]. In veterinary medicine, it has been reported that canine serum ferritin levels are increased in lymphoma, histiocytic sarcoma and immune mediate hemolytic anemia (IMHA) [3, 7, 10].

From past reports, detection of serum ferritin in dogs has been accomplished by sandwich enzyme-linked immunosorbent assays (ELISAs) using a mouse anti-canine ferritin monoclonal antibody, and purified canine liver ferritin as the standard [1]. However, it has been reported that in canine serum an autoantibody exists that binds to ferritin. This is thought to be one of the factors confounding accurate detection and measurement of serum ferritin by immunological techniques. We have developed a new homologous sandwich ELISA that is able to reliably detect and measure canine serum ferritin levels, and we assessed its accuracy.

Ferritin purified from canine heart tissue and anti-canine heart ferritin antibodies were produced using techniques reported previously [13, 14]. The concentration of purified ferritin and anti-ferritin antibody was determined by the methods of Lowry et al. with bovine serum albumin used as a protein standard [8].
We examined the specificity of the antibody using immunoprecipitation and immunoblotting techniques as reported previously [13]. The antibody we used recognized both the H and L subunits of canine serum ferritin (Fig. 1).

For the sandwich ELISA, 400 ng/ml purified rabbit anti-canine heart ferritin antibody diluted in phosphate-buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.2) was added to each well of 96-well microtiter plates (Immuno Plate Maxisorp F96; Nunc, Roskilde, Denmark), and stored overnight at 4°C. The antibody-coated plates were then washed with PBS containing 0.05% (v/v) Tween 20 (PBST). ELISA buffer (PBS containing 0.1% gelatin and 0.1% Tween20) was added to each well, incubated for 1 hr at room temperature, and then washed once with PBST. Purified canine heart ferritin was used as a standard, and serially diluted 2-fold from 200 to 3.125 ng/ml in ELISA buffer. Samples added to wells were either canine sera (100 μl) diluted with dilution buffer (ELISA buffer containing 0.5 M ammonium sulphate, pH 7.2) or standards (100 μl). Plates were incubated at 37°C for 2 hr and then washed three times with PBST. Following the wash steps, 100 μl of 200 ng/ml alkaline phosphatase-conjugated rabbit anti-canine heart ferritin [2] was added to each well. Plates were incubated at 37°C for 3 hr. After three times washing with PBST, color development was performed as previously described [11], and the absorbance at 405 nm was measured (iMark, Bio Rad, Tokyo, Japan). The standard curve for this ELISA is shown in Fig. 2.

For the dilution tests, two serum samples were used; they were diluted 50- to 400-fold with dilution buffer. For both samples, no dilution effects were observed (Fig. 3). Intra-assay variability was assessed using 12 samples, which were applied to the sandwich ELISA and measured three times on the same day. The coefficients of variation for the 12 samples were 0.6–2.2%. Inter-assay variability was tested using
four samples over three different days. The coefficients of variation for these samples were 3.2–10.9%. The higher coefficients of variation for inter-assay variability were likely caused by adjustments to the standard solution and dilution of the samples. Recovery of canine heart ferritin, which had been added to diluted canine serum, ranged from 96–102% (Table 1). These results showed that our sandwich ELISA was reliable for detection and measurement of canine serum ferritin.

Clinically healthy dogs (n = 163) were selected from those that visited Tomizawa Animal Hospital between 2010 and 2011. This study was approved by the Kitasato University Animal Committee. Dogs (1–16 year-old, mean 4 years) were randomly selected (male, n = 82; female, n = 81). The most common breeds were miniature dachshund (n = 32; 19.6%), mongrel (n = 27; 16.6%) and shibas (n = 20; 12.3%). Age, gender and breed did not appear to have an effect on serum ferritin concentration.

Blood (2 ml) was sampled from the cephalic or jugular vein, left to coagulate at room temperature for 30 min and then centrifuged (1,640 × g, 5 min) to separate the serum. Serum samples were stored at -20°C until required.

In our sandwich ELISA, serum ferritin concentration in 163 healthy animals was 789 ± 284 ng/ml (mean ± SD; range 261–1,889 ng/ml). The mean (value, range) serum ferritin concentrations determined previously in normal dogs by Andrews [1] and Watanabe [14] were 252 ng/ml (n = 61, 80–800 ng/ml) and 479 ng/ml (n = 51, 298–959 ng/ml), respectively. The values reported in this study are obviously much higher. This is possibly due to the reactivity between the ferritin subunit and the antibody used in the ELISA. The ferritin protein is composed of two subunits (H and L) that form a 24-mer. Immunological differences between the ferritin subunits have been previously reported [9]. The antibodies Andrews and Watanabe used were a mouse anti-canine
ferritin monoclonal antibody and a rabbit anti-rat liver ferritin polyclonal antibody. It was believed that the reactivity to both H and L subunits was significantly different to the rabbit anti-canine heart ferritin polyclonal antibody we used in our study. When the subunit composition ratio of a measured sample differs greatly from a standard, it may influence the results; however, we did not examine this point.

In this study, we attempted to develop a sandwich ELISA, using a homologous antibody, with greater precision than that used by other researchers in the past. Our sandwich ELISA demonstrated high levels of confidence, and we believe it can be adapted for use in the measurement of canine serum ferritin levels.

Fig. 1. Immunoblot detection of canine serum ferritin. Lane 1: marker protein; 2: purified canine heart ferritin (2 μg); 3: purified canine liver ferritin (2 μg); 4: 2 ml of normal canine serum subjected to immunoprecipitation using 5 μg of rabbit anti-rat liver ferritin polyclonal antibody. H (21.1 kDa) and L (18.7 kDa) ferritin subunits are indicated by arrows. The white arrow indicates the likely heavy chain of the rabbit anti-rat liver ferritin antibody collected for immunoprecipitation.

Fig. 2. Standard curve for our canine heart ferritin sandwich ELISA. Purified canine heart ferritin was serially diluted 2-fold with ELISA buffer from 400 to 3.125 ng/ml. The values indicate the means measured in triplicate, and the error bars show standard deviation.

Fig. 3. Effects of serum dilution on the sandwich ELISA. Two serum samples were diluted 50-, 100-, 200-, and 400-fold with dilution buffer. Values show the mean
measured in triplicate, and error bars indicate standard deviation. There was a strong linear relationship ($R^2>0.99$) between serum dilution and ferritin concentration.

Table 1. Recovery of canine heart ferritin from canine sera. A known concentration of purified canine heart ferritin (5 and 10 ng/ml) was added to three canine serum samples and then measured by ELISA before recovery from the serum sample.
REFERENCES


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