〈Research Article〉

Development of canine serum ferritin assay using latex immunoturbidimetric human ferritin assay

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Summary The aim of this study was to establish a canine serum ferritin assay using a commercial latex immunoturbidimetric human ferritin assay. Canine heart ferritin and liver ferritin with 3.69 and 0.43 H/L subunit ratios, respectively, was added to serum, and recoveries of canine heart and liver ferritins added were 60–111% (heart: 60–111%; liver: 85–103%). In contrast, agglutination activity of the heart ferritin was 7.6-fold lower than that of the liver ferritin, which corresponded to almost the same activity as that of commercial human ferritin antigen. The detection limit of canine liver ferritin was 6.6 ng/mL and there was a linear relationship between serum dilution and ferritin concentration from no dilution to 1:10 dilution. The coefficient variances of serum ferritin determination were 1.8% (n=3) and 1.3% (n=5) in intra- and inter-assays, respectively. Serum samples stored at –20˚C did not influence serum ferritin concentrations, but the serum stored at room temperature for over 1 week and over two weeks at 4˚C had increased ferritin concentrations. Heparin (<43 U/mL) and EDTA (<22 mM) as anticoagulant reagents did not affect serum ferritin levels. Addition of hemoglobin (<500 mg/dL), Intralipos® (<2.5%), and conjugated (<43 U/mL) and free bilirubin to serum samples did not affect serum ferritin levels. This assay showed reference values of 43–311 ng/mL (mean: 146 ng/mL) from 349 healthy canine sera. Centrifugation (5 min, 14,000 × g) of serum samples showed a maximum 24% lower ferritin concentration as compared to that in untreated samples, suggesting that anti-ferritin autoantibody forms an immune complex with serum ferritin. These data are applicable to the measurement of ferritin in the serum of canines with the latex immunoturbidimetric human ferritin assay.

Key words: Canine, Heart ferritin, Latex immunoturbidimetric assay, Liver ferritin, Serum ferritin.

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Received for publication: February 7, 2022
Accepted for publication: March 24, 2022

1. Introduction

Iron is an essential transition element for living organisms and is required for redox reactions in various metabolic processes, such as electron transport, the citric acid cycle, and DNA synthesis[1]. Ferritin functions as an iron reservoir, which is a 24-mer protein composed of two subunits, namely H (heavy or heart) and L (light or liver). The ferroxidase of the H subunit oxidizes ferrous iron to ferric iron for iron uptake. The L subunit lacks the conserved amino acids associated with ferroxidase activity but contributes to ferritin stability and tends to store iron for a long time[1]. Amino acid sequence homologies between mammalian H and L subunits are low (50–56%), and the H and L subunits are immunologically different from each other[1].

Ferritin is a ubiquitous protein, but ferritin circulates at a relatively low level (<1 μg/mL). Serum ferritin levels are the best indicator for estimating body iron stores in animals, such as humans, horses, cattle, pigs, canines, and felines[1,2], whereas serum ferritin is a marker for malignant and inflammatory conditions[1,3]. The L subunit is predominant in ferritin from human and bovine fetal sera[1], while canine serum ferritin is H subunit-rich[1]. Ferritin-binding proteins (FBPs) in mammalian circulation systems have been identified, including H-kininogen, alpha-2-macroglobulin, autoantibodies, apolipoprotein B, and fibrinogen[4]. These proteins are involved in the rapid clearance (T1/2<10 min) of circulating ferritin through indirect receptor-mediated uptake by forming complexes with them[4].

In equine, the inhibitory effect of fibrinogen on the serum ferritin assay was observed. Anti-ferritin autoantibodies and alpha-2-macroglobulin have been identified as common mammalian FBPs[1,4]. However, canine anti-ferritin autoantibody was identified as an FBP, but an inhibitory effect of canine serum on the ferritin assay was not observed because of the higher affinity of anti-canine liver ferritin antibodies, used by the enzyme linked immunosorbent assay (ELISA) method, than that of anti-ferritin autoantibody for canine ferritins[5]. In contrast, canine anti-ferritin autoantibody tends to recognize more H subunit-rich isoferritins than L subunit rich isoferritins[4].

For humans, an automated latex immunoturbidimetric human ferritin assay kit (FER-Latex method; FER-Latex(X2)CN SEIKEN, DENKA Seiken, Japan] is commercially available[6]. However, canine serum ferritin assay kits are not commercially applicable for clinical survey, although some ELISA kits are commercially available. This study provides preliminary data for the development of a canine serum ferritin assay using FER-Latex method, using H subunit predominant canine heart ferritin in place of H subunit predominant canine serum ferritin[7] and anti-ferritin autoantibodies. Finally, we recommend a canine serum ferritin assay using the latex immunoturbidimetric human ferritin assay kit with supplemented human ferritin antigen, which had the same agglutinin activity as canine liver ferritin, because canine heart ferritin showed lower agglutinin activity in the same assay, and the underestimation of serum ferritin concentrations by anti-ferritin autoantibody was negligible.

2. Materials and Methods

Materials

A FER-Latex(X2)CN SEIKEN was purchased from DENKA Seiken (Goizumi, Niigata 959-1695, Japan). Canine hemoglobin (Hb) was obtained from Kitayama Labes Co., Ltd. (Ina, Nagano, Japan). Supplies of conjugated and free bilirubin (Interference Check A) were purchased from Sysmex Co. (Kobe, Hyogo Japan). Intralipos® was purchased from Otsuka Pharmaceutical Industries, Inc. (Chiyoda, Tokyo, Japan). Vacuum blood collection tubes containing heparin and EDTA were purchased from FUJIFILM VET Systems Co., Ltd. (Mitaka, Tokyo, Japan) and Kyokuto Pharmaceutical Industries, Ltd. (Chuo, Tokyo, Japan).

Canine serum

Canine sera were collected from canines brought into FUJIFILM VET Systems Co., Ltd. (Mitaka, Tokyo Japan) for medical examination, and the samples were confirmed to have no biochemical

abnormalities. All experiments were conducted following the established guidelines for animal welfare and were approved by the Committee on the Ethics of Animal Experiments of Kitasato University (permit no.: 20-048).

Ferritin preparation

Canine heart and liver ferritin were purified from pieces of frozen canine heart and liver as described previously[8]. SDS-PAGE was performed according to the method described by Schägger and von Jagow[9], using 4.5% polyacrylamide stacking gel and 10% polyacrylamide running gel, followed by staining with Coomassie Brilliant Blue R250. Densitometry of stained ferritin subunit bands was performed at 565 nm using a Flying Spot Scanner (Shimadzu Model CS-9000) (Shimadzu, Kyoto, Japan).

Ferritin measurement (FER-Latex method)

A canine serum ferritin assay was carried out with FER-Latex method using a method similar to that used for the FER-Latex method according to the manufacturer's protocol. Briefly, the diluent consisted of a serum sample (5 μL) diluted approximately with 0.9% NaCl, R-1 reagent (50 μL, 170 mM glycine/HCl, pH 7.3), and R-2 reagent (25 μL, suspension of polystyrene latex particles in R-1) was applied to BioMajesty 6070 G (Japan Electron Optics Laboratory Co., Ltd., Akishima, Tokyo, Japan). In the recovery test, serum samples diluted approximately with 0.9% NaCl were added to the tenth volume of known amounts of purified canine heart and liver ferritin, and the recovery of ferritin added to serum samples was measured by FER-Latex method using the human ferritin standard. Serum samples were subjected to the ferritin assay after the addition of heparin (13–43 U/mL) and EDTA (6.5–22 mM) by changing the serum amount added. Canine hemoglobin (50–500 mg/dL), Intralipos® (0.25–2.5%), and conjugated (2.5–25 mg/dL) and free bilirubin (2.5–25 mg/dL) were added to serum samples after diluting by 2-fold staircase dilution with 0.9% NaCl. Additionally, after centrifugation of serum samples at 14,000 × g for 5 min, the supernatant obtained was applied to a latex immunoturbidimetric human ferritin assay.

Statistical analysis

The reference value of serum ferritin concentrations was determined by the Robust Z-Score method. Microsoft® Excel® for Microsoft 365 MSO was used for data analysis.

3. Results

Comparison between canine heart and liver ferritin levels via canine serum ferritin assay using the FER-Latex method

A previous study showed that the molecular masses of the H and L subunits of both heart and liver ferritins were 21.1 and 18.7 kDa, respectively, and the H/L subunit ratios of heart and liver ferritins were 3.69 and 0.43, respectively[7]. The recoveries of purified canine heart and liver ferritin added to two serum samples were separately measured (heart ferritin: 60–111%; liver ferritin: 85–103%) (Tables 1 and 2). Although there was linearity (heart: r=0.999; liver: r=0.995) between agglutination activity of the ferritin measurement and it's protein amount in both canine heart and liver ferritins (Fig. 1), each 1 μg of ferritin protein showed immuno-cross-reactivity of 0.12 and 0.91 μg in heart and liver ferritins, respectively, indicating that canine heart ferritin was 7.6-fold less immunoreactive than canine liver ferritin, and agglutinin activity of canine liver ferritin was almost equal to the human ferritin standard supplied with the assay kit. Estimated serum ferritin concentrations of 10 canine sera, including two sera (Nos. 1 and 2) of (Table 2), ranged from 334 to 2,080 ng/mL (data not shown) based on calculations with the human ferritin standard from the lower immuno-cross-reactivity of canine heart ferritin, which were remarkably high compared to previous data (80–800 ng/mL) by Andrews et al. (1994)[10]. Therefore, in this study, the canine serum ferritin assay was performed using the human ferritin standard supplemented in place of canine heart and liver ferritin with FER-Latex method. There was linearity (r=0.999) between

Table 1 Recoveries of known amounts of canine liver ferritin added to two canine sera

Serum No.	Added ferritin (ng/mL)[1)]	Founded ferritin (ng/mL)[1)]	Recovery (%)
	0	37	
1	163	175	85
	326	373	103
	0	121	
2	163	278	96
	326	456	103

1) Ferritin concentrations were determined by latex immunoturbidimetric human ferritin assay kit with human ferritin antigen as a standard protein.

Table 2 Recoveries of known amounts of canine heart ferritin added to two canine sera

Serum No.	Added ferritin (ng/mL)[1)]	Founded ferritin (ng/mL)[1)]	Recovery (%)
	0	140	
1	10	146	60
	19	152	63
	0	175	
2	10	186	110
	19	196	111

1) Ferritin concentrations were determined by latex immunoturbidimetric human ferritin assay kit with human ferritin antigen as a standard protein.

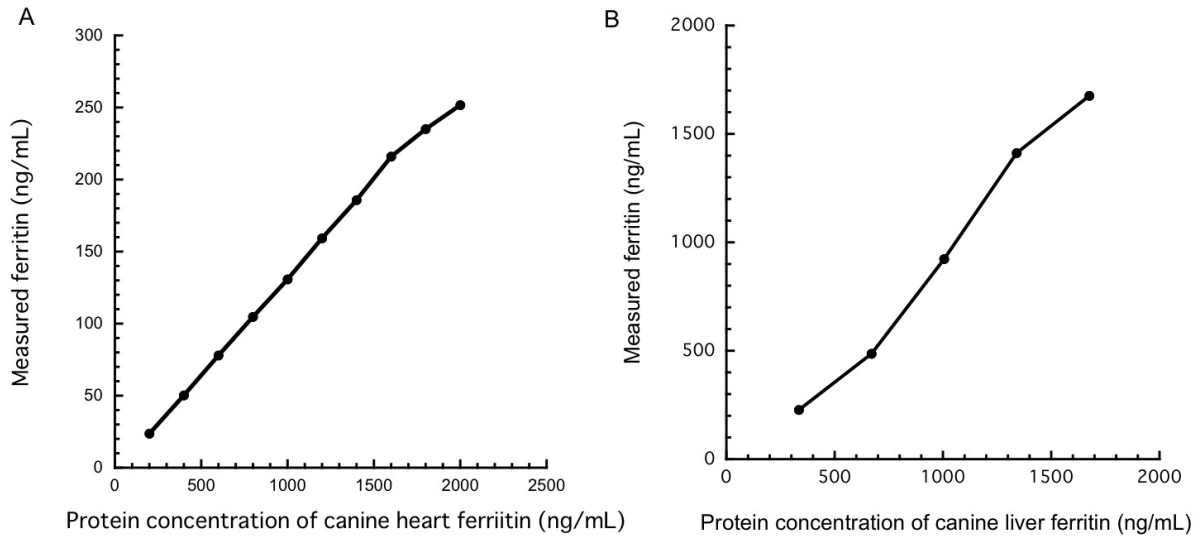

Fig. 1. Linearity between ferritin concentration and its protein concentration in canine heart and liver ferritins. Protein concentrations of canine heart (A) and liver ferritins (B) were determined using a BCA assay kit with BSA as a standard. These ferritin samples were subjected to a latex immunoturbidimetric human ferritin assay.

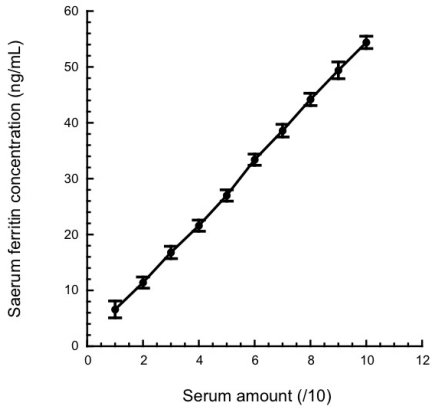

Fig. 2. Linearity between ferritin concentration and serum amount. Serum without dilution was originally used for latex immunoturbidimetric human ferritin assay followed by a decrease of every tenth volume until a tenth of its original volume. Data represent mean ± SD (n=5).

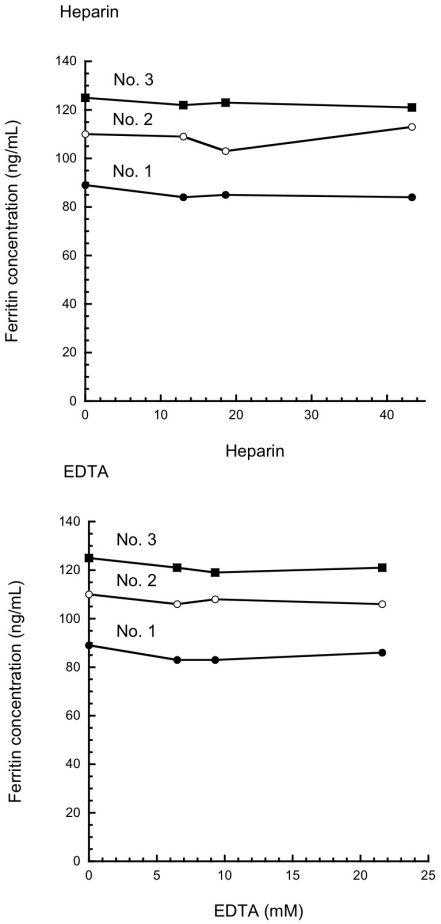

Fig. 4. Effects of heparin and EDTA on canine serum ferritin assay. Serum samples (No. 1–3) were subjected to latex immunoturbidimetric human ferritin assay in the presence of heparin and EDTA as anticoagulants, whose concentrations were adjusted by changing the serum volume.

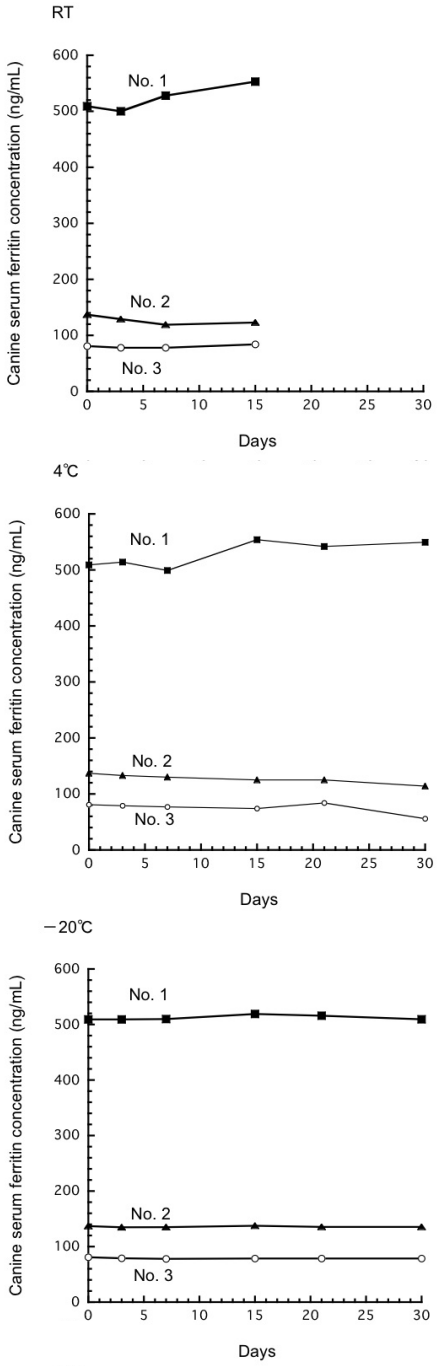

Fig. 3. Effects of storage of three serum samples with high, middle, and low ferritin concentrations at room temperature, 4˚C, and –20˚C on their respective serum ferritin concentrations. Each serum sample with high (No. 1), middle (No. 2), and low (No. 3) ferritin concentrations with about 500, 130, and 80 ng/mL, respectively, were stored at room temperature of 4˚C and –20˚C during certain periods, followed by ferritin determination by latex immunoturbidimetric human ferritin assay.

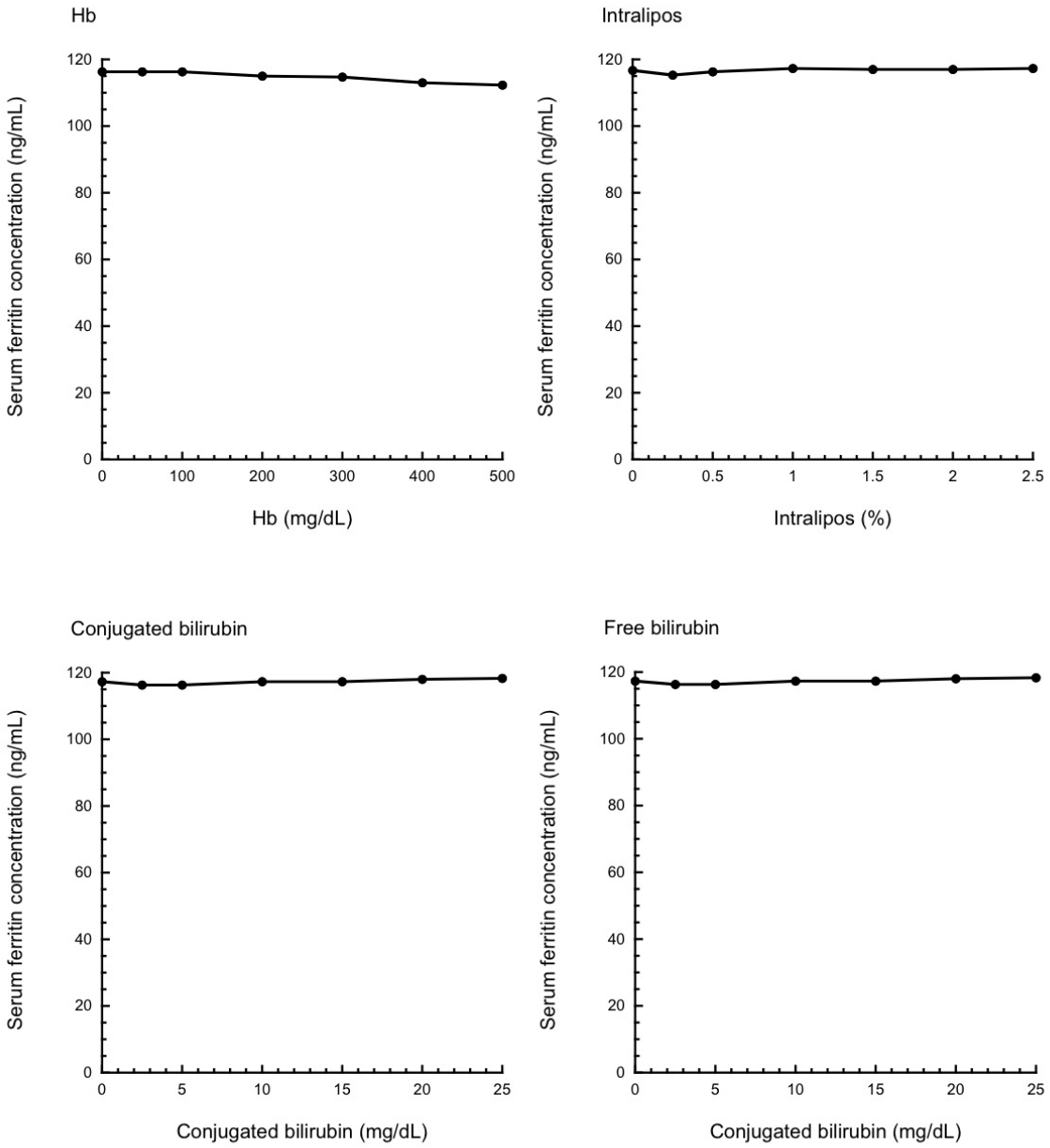

Fig. 5. Effect of Hb, Intralipos®, and conjugated and free bilirubin on canine serum ferritin assay. Hb, Intralipos®, and conjugated and free bilirubin were diluted by 2-fold staircase dilution with 0.9% NaCl from each original concentration (Hb: 500 mg/dL; Intralipos®: 2.5%; conjugated bilirubin: 25 mg/dL, and free bilirubin: 25 mg/dL) as indicated in Fig. 5.

serum ferritin concentration and serum amount (from no dilution to 10-fold dilution), which indicated that the assay limit was at least 6.6 ng/mL (Fig. 2).

Ferritin determination of canine serum ferritin assay using FER-Latex method

This study examined the effect of temperature of stored serum samples on ferritin assays using three serum samples with high, medium, and low serum ferritin concentrations of 500, 130, and 80 ng/mL, respectively (Fig. 3). Storage of serum samples at –20˚C did not influence serum ferritin levels. On the other hand, high serum ferritin concentration was mildly affected, i.e., a slight increase in ferritin concentration was observed, after storage at room temperature for over one week and at over 4 ºC for two weeks. The serum samples with different ferritin concentrations were not affected by heparin (<43 U/mL) or EDTA (<22 mM) (Fig. 4). Serum ferritin

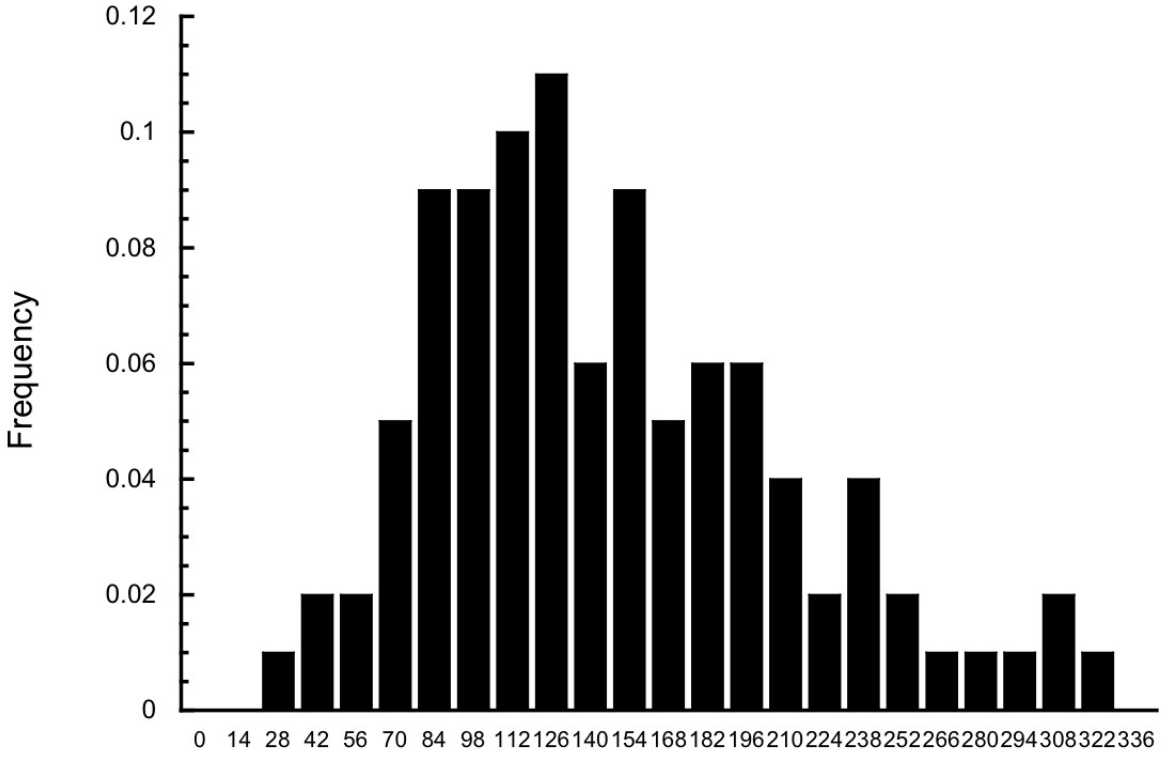

Fig. 6. Robust analysis of serum ferritin concentrations from 349 healthy canines. Reference values of serum ferritin concentrations were from 43 to 311 ng/mL as indicated with the dotted line.

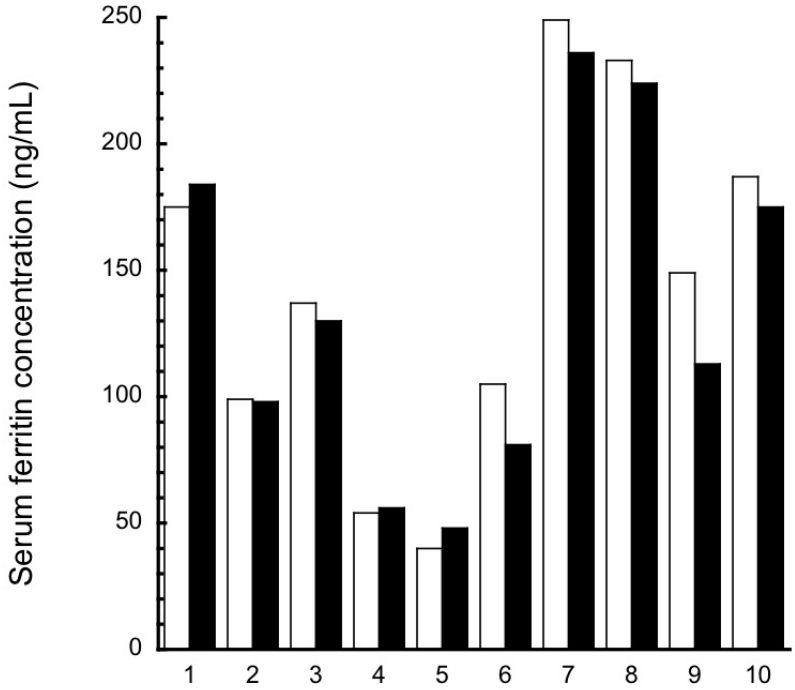

Fig. 7. Effect of centrifugation on serum ferritin concentration. Ten serum samples were centrifuged for 5 min at 14,000 × g, and the supernatant samples (solid bar) obtained were subjected to latex immunoturbidimetric human ferritin assay together with untreated samples (open bar).

concentration (<120 ng/mL) was also not affected by hemoglobin (<500 mg/dL), Intralipos® (<2.5%), and conjugated and free bilirubin (<25 mg/dL in both conditions) (Fig. 5). Intralipos® (10 and 20%) is used as a lipid emulsion for peripheral nutrition, and the residual fat emulsion in the circulation does not likely affect the ferritin concentration via slow infusion (15-20 mL/h)[11]. Ferritin reference values from serum samples from 349 healthy canines ranged from 43 to 311 ng/mL (Fig. 6), in accordance with previous data (80–800 ng/mL) by Andrews et al. (1994)[10]. From these results, intra- and inter-assays of low ferritin concentration (81 ng/mL) in canine serum were 1.8% (n=3) and 1.3% (n=5), respectively.

Effect of centrifugation of serum samples on serum ferritin concentration

Canine serum contains autoantibodies against ferritin to be immunoprecipitated by Earth's gravity[12]. This study showed serum ferritin concentrations before and after centrifugation for 5 min at 14,000 × g (Fig. 7). Serum Nos. 6 and 9 showed 23% and 24% lower ferritin concentrations, respectively, than the untreated sample.

4. Discussion

Canine serum ferritin predominates the H subunit (H/L subunit ratio: 3.46±1.12, n=6)[7]. In this study, canine heart and liver ferritins with H/L subunit ratio of 3.69 and 0.43, respectively, were used to establish a canine serum ferritin assay with a latex immunoturbidimetric human ferritin assay kit. Ferritin recoveries of canine heart and liver ferritin added to two serum samples were 85–111% except for one serum sample showing low recovery (60–63%) of canine heart ferritin. In contrast, immune-cross-reactivity of canine heart ferritin was about 7.6-fold lower than that of canine liver ferritin, showing almost the same immune-cross-reactivity with human ferritin as an antigen in the latex immunoturbidimetric human ferritin assay kit, as shown in Fig. 1. Rabbit anti-canine liver ferritin antibody showed approximately 2-fold lower immune-cross-reactivity to canine heart ferritin than to homogeneous canine liver ferritin[8]. Due to H subunit-rich serum ferritin[7,12], the use of H subunit-predominant canine heart ferritin as a ferritin standard and/or anti-H subunit antibody is suitable for measurement in a canine serum ferritin assay. However, two ELISA kits (BLUEGENE: Ca FE ELISA kit; Signalway Antibody: Dog Ferritin heavy chain ELISA kit) for ferritin or heart type of canine ferritin did not fully show immunoreactivity with canine serum ferritin as compared to serum ferritin concentrations measured by FER-Latex method with the human ferritin standard for unknown reasons. However, in this study, serum ferritin concentrations were remarkably high as calculated from the ferritin standard from untreated serum samples (Table 2); we observed 7.6-fold lower immunoturbidimetric activity of canine heart ferritin. Autoantibodies, probably IgM, against ferritin in canine serum showed more immunoreactivity to the H subunit than to the L subunit[4], suggesting that the ferritin concentration in canine sera results in an inconsistent higher serum ferritin concentration by increasing immunoturbidity, as canine heart ferritin was used as a standard instead of the human ferritin standard. Canine autoantibody to ferritin causes immunoprecipitation after forming an immune complex with ferritin in the canine serum by Earth's gravity, as described above. Amino acid sequence homologies between mammalian ferritin H and L subunits are low (50–56%)[1]. Further studies are needed to clarify the effect on canine ferritin immunoassays, such as latex immunoturbidimetric assays, when heart ferritin is used as an antigen and standard. Amino acid sequence homology human and canine L subunits is 86%[1], and canine liver ferritin showed the same latex agglutinin activity as the human ferritin standard. Therefore, this study investigated the applicability of a canine serum ferritin assay with a commercial FER-Lates method using human ferritin standards in place of canine liver ferritin.

The assay limit of the canine serum ferritin assay was 6.6 ng/mL. Serum samples can be stored at room temperature for approximately one week. Anticoagulants, such as heparin (<43 U/mL) and

EDTA (<22 mM) did not affect the canine serum ferritin assay, indicating that canine plasma is also available for ferritin assay. Hb and conjugated and free bilirubin as interferes did not affect this assay. Intralipos® (<2.5%) as an intravenous fluid infusion did not affect this assay. The reference value of canine serum ferritin concentration ranged from 43 to 311 ng/mL, differing from previous data (80 to 800 ng/mL) by Andrews et al. (1992)[10]. This FER-Latex method for the canine serum ferritin assay is also suitable according to the data regarding assay limit (6.6 ng/mL) and serum dilution (no dilution).

The presence of autoantibodies to ferritin in the serum of canines may interfere with the ferritin assay. The maximum effect of high-speed centrifugation (14,000 x g) on canine serum ferritin assay was observed as 24% lower ferritin concentration than that without centrifugation, suggesting that the immune complex between auto-ferritin antibody and serum ferritin was precipitated with centrifugal force. The reference value of this canine serum ferritin assay ranged from 43 to 311 ng/mL. This reduction due to centrifugation is not likely to affect ferritin measurements from the clinical point of view, as serum ferritin concentrations after centrifugation conservatively range from 57 to 409 ng/mL.
The latex immunoturbidimetric ferritin assay kit is a simple method without the need for dedicated instrumentation, and this study demonstrates its applicability to canine serum ferritin assay using the latex immunoturbidimetric human ferritin assay.

Conflicts of interest

The authors declare no conflicts of interest.

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