Spectrophotometry and Ultracentrifugation for Measurement of Plasma Lipids in Dogs with Diabetes Mellitus

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Background: There are conflicting reports of plasma lipoprotein lipid content in dogs with diabetes mellitus (DM).

Objectives: To determine lipoprotein lipid content of plasma of dogs with DM by spectrophotometry and ultracentrifugation; to compare lipoprotein lipid content in diabetic and healthy dogs; and to quantify apolipoprotein B-100 (ApoB) in dogs with DM.

Animals: 22 dogs with DM and 9 healthy dogs.

Methods: Cross-sectional study. Triglyceride (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL-C) concentrations were measured by spectrophotometry. Very low-density lipoprotein cholesterol (VLDL-C) and low-density lipoprotein cholesterol (LDL-C) concentrations were calculated after ultracentrifugation. Non-HDL-C cholesterol was calculated by subtracting HDL-C from TC. ApoB was quantified by ELISA. The Mann-Whitney test was used for comparison of median lipoprotein concentrations, and Spearman’s correlation was used to assess associations between ApoB and lipoprotein fractions.

Results: All values are reported in mg/dL. Median TG (122), TC (343.5), HDL-C, (200), VLDL-C, (27) LDL-C (68), non-HDL-C (114), and ApoB (320) were significantly higher in dogs with DM, compared to healthy dogs (57, 197, 168, 12, 16, 31, and 258, respectively, P-values 0.0079, <0.001, 0.029, 0.011, <0.001, <0.001, 0.025, respectively). A significant association was found between ApoB and LDL-C (Spearman’s rho = 0.41, P = 0.022) and between ApoB and non-HDL-C (Spearman’s rho = 0.40, P = 0.027).

Conclusions and Clinical Importance: Dyslipidemia of dogs with DM is characterized by pronounced increases in LDL-C and non-HDL-C concentrations, although all lipoprotein fractions are significantly increased. Knowledge of specific lipoprotein fraction alterations in dogs with DM can enhance treatment options for diabetic dyslipidemia in dogs.

Key words: Apolipoprotein B; Cholesterol; Dyslipidemia; Lipoprotein; Triglycerides.

Hypercholesterolemia is common in dogs with diabetes mellitus (DM). However, the specific composition of lipoprotein fractions contributing to this dyslipidemia is not well characterized. One study utilizing agarose gel electrophoresis reported that total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG) concentrations were all increased in 9 dogs with DM compared to healthy dogs. Another study, employing ultracentrifugation and agarose gel electrophoresis, reported that TC, very low-density lipoprotein cholesterol (VLDL-C), and HDL-C concentrations were increased, but no significant increase in LDL-C concentration was noted in 11 dogs with DM, compared to healthy dogs. A third study reported that in 6 dogs with DM in which lipoproteins were quantified by agarose gel electrophoresis, LDL and VLDL concentrations were higher, whereas HDL concentration was lower compared to healthy dogs. These study-dependent differences in lipoprotein fraction concentrations could be attributed to the use of different methodologies and small study sample sizes. In healthy dogs, 8 years of age or older, TC and LDL-C concentrations are higher, whereas HDL-C concentration is lower compared to younger dogs. Therefore, LDL-C concentration in healthy dogs is also increased with obesity and is influenced by sex, neuter status, and breed.

LDL-C concentration is an important predictor of cardiovascular disease in humans. However, LDL-C reflects only the total amount of cholesterol carried by LDL. In contrast, apolipoprotein B-100 (ApoB), a single large protein molecule that carries LDL lipids, concentrations were increased, but no significant increase in LDL-C concentration was noted in 11 dogs with DM, compared to healthy dogs. A third study reported that in 6 dogs with DM in which lipoproteins were quantified by agarose gel electrophoresis, LDL and VLDL concentrations were higher, whereas HDL concentration was lower compared to healthy dogs. These study-dependent differences in lipoprotein fraction concentrations could be attributed to the use of different methodologies and small study sample sizes. In healthy dogs, 8 years of age or older, TC and LDL-C concentrations are higher, whereas HDL-C concentration is lower compared to younger dogs. Therefore, LDL-C concentration in healthy dogs is also increased with obesity and is influenced by sex, neuter status, and breed.

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estimates the number of LDL particles. It is possible that the number of LDL particles is more important than LDL-C concentration in predicting cardiovascular disease in humans.\textsuperscript{8,9} Although several methods for LDL particle number measurement have been published, quantification of ApoB is recommended because it is as effective as other methods in predicting cardiovascular disease, and assays for ApoB measurement are readily available and easy to use.\textsuperscript{10} Non-HDL-C concentration is another predictor of cardiovascular disease in humans and is calculated by subtracting HDL-C concentration from TC concentration. Some studies suggest that non-HDL-C is a better marker for cardiovascular disease than LDL-C.\textsuperscript{9} In humans with type 1 diabetes, non-HDL-C and ApoB are correlated, but are considered to be complimentary predictors of cardiovascular disease.\textsuperscript{10}

Dogs with DM are at increased risk of atherosclerosis compared to dogs without DM.\textsuperscript{11} However, ApoB has not been reported in dogs with naturally occurring DM. The goals of this study were therefore to determine lipoprotein lipid content in dogs with DM by spectrophotometry and ultracentrifugation, to compare lipoprotein lipid content of dogs with DM with those of healthy dogs of similar age and neuter status, and to quantify ApoB in dogs with DM. Knowledge of the specific lipoprotein lipid alterations in dogs with DM can promote effective therapeutic interventions for diabetic dyslipidemia in dogs.

Materials and Methods

A cross-sectional study was performed. Client-owned, insulin-treated dogs with DM, examined at the Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania (MJR-VHUP) for routine outpatient serial measurement of glucose concentrations, were prospectively enrolled between October 20, 2014, and November 30, 2015, at research designated times of one of the authors (ECS). Inclusion criteria were a diagnosis of insulin-treated DM, the dog's availability for blood draw after an 8- to 12-hour fast, and willingness of the owner to sign an informed consent and have the dog participate in the study. A diagnosis of DM was based on presence of characteristic clinical signs (polyuria, polydipsia, polyphagia, or weight loss), persistent hyperglycemia (serum or plasma glucose concentration greater than 250 mg/dL), and glucosuria. Exclusion criteria were a body weight less than 4 kg, due to risks of blood sampling associated with small body size, diabetic ketoacidosis, hyperosmolar hyperglycemic syndrome, vomiting, diarrhea, decreased appetite, or any new or unusual clinical sign that indicated a change in health status. Dogs were enrolled into the study regardless of the diet they were eating, the insulin product used, insulin dose, duration of insulin treatment, administration of additional drugs, presence of concurrent disease, ability to perform or review other diagnostic test results, or blood glucose concentrations noted on serial blood glucose measurements. The Institutional Animal Care and Use Committee at the University of Pennsylvania approved the study, and all owners provided written informed consent at the time their dog was enrolled into the study. Healthy control dogs were recruited from the population of dogs owned by MJR-VHUP staff and students. Screening of control dogs included history, physical examination, complete blood count, and serum biochemistry panel.\textsuperscript{9,8} Inclusion criteria for the control dogs were at least 8 years of age, normal history and physical examination, and normal complete blood count and serum biochemistry panel findings. As with dogs with DM, inclusion of healthy dogs required availability for a blood draw after an 8- to 12-hour fast, and willingness of the owner to sign an informed consent. Dogs were excluded from the control group if they were not neutered or if they were receiving any medication other than preventive medication for parasitic infestation.

Physical examination findings including body condition score (BCS), diet, and clinical signs were recorded in all dogs. BCS of 1 (emaciated) to 9 (severely obese) was subjectively assigned to each dog by 1 investigator (ECS).\textsuperscript{12} The type and dose of insulin, as well as serial plasma or serum glucose concentrations measured every 2 hours over an 8- to 12-hour period after insulin administration and feeding on the day of enrollment into the study, were recorded in dogs with DM.\textsuperscript{8} Blood samples for lipid analysis were obtained from dogs with DM at the time that venipuncture was performed for the purpose of blood glucose measurement after an 8- to 12-hour fast, just before the dog was discharged from the hospital. In control dogs, a single venipuncture was performed to collect blood for the complete blood count, biochemistry panel, and lipid analysis after an 8- to 12-hour fast. If results of the complete blood count and biochemistry panel were normal in healthy dogs, the blood sample was submitted for lipid analysis.

For the purpose of lipoprotein fraction measurements, 4 mLs of blood were collected via standard venipuncture technique and placed directly in a 4-mL EDTA tube.\textsuperscript{3} Within 15 minutes of venipuncture, the blood samples were centrifuged at room temperature at 3,200 rpm for 3 minutes, as per standard clinical laboratory procedure. The plasma was then separated and stored in a 2-mL cryovial via 4°C at MJR-VHUP for up to 72 hours.\textsuperscript{5} Samples were walked to the Translational Core Laboratory at the Smilow Center for Translational Research at the Perelman School of Medicine, University of Pennsylvania, twice weekly for lipid analysis. Upon delivery to the Translational Core Laboratory, plasma TG, TC, and HDL-C concentrations were measured with the Roche/Hitachi Cobas C311 biochemical analyzer and utilizing enzymatic colorimetric tests and spectrophotometry.\textsuperscript{4} All reagents were provided by the manufacturer, and manufacturer protocols were followed as described here. For plasma TG quantification, lipoprotein lipase was used to hydrolyze TG to glycerol. Glycerol was then oxidized to hydrogen peroxide, which reacts with 4-amino-antipyrine and sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline to form a purple-blue dye, the color intensity of this dye is measured photometrically and reflects the TG concentration. For plasma TC quantification, cholesterol esterase was used to hydrolyze cholesterol esters to form free cholesterol, which was then oxidized to form hydrogen peroxide. The hydrogen peroxide reacts with phenol and 4-aminophenazone to form a red quinone-imine dye. The color intensity of this dye is directly proportional to TC concentration. Before plasma HDL-C measurement, dextran sulfate (in the presence of magnesium) forms water-soluble complexes with LDL, VLDL, and chylomicrons. These complexes are resistant to polyethylene glycol-modified cholesterol esterase. However, HDL-C esters are broken down by polyethylene glycol-modified cholesterol esterase to produce HDL-C, which is further oxidized to produce hydrogen peroxide. The hydrogen peroxide reacts with 4-amino-antipyrine and sodium N-(2-hydroxy-3-sulfo-propyl)-3,5-dimethoxyaniline to form a purple-blue dye, the color intensity of which is directly proportional to HDL-C concentration. After these 3 plasma lipid measurements, plasma was centrifuged in the Beckman LE-50 Ultracentrifuge at a rotor speed of 92,500 g for 18 hours at 10°C to float plasma density <1.006 g/mL, the density which corresponds to VLDL in dogs.\textsuperscript{3,5} Cholesterol was then measured in the fraction with density >1.006 g/mL, which contains LDL and HDL. VLDL-C was calculated by subtracting the cholesterol measured in the fraction with density >1.006 g/mL (representing both LDL-C and HDL-C) from plasma
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TC, LDL-C was calculated by subtracting plasma HDL-C from the cholesterol fraction with density >1.006 g/mL (representing both LDL-C and HDL-C). Non-HDL-C cholesterol was calculated mathematically by subtracting HDL-C from TC. Plasma remaining after this initial analysis was stored at −80°C for ApoB quantification by a commercial canine validated ELISA. The minimum detectable ApoB concentration with this assay is <0.002 mg/dL. Intra-assay and interassay coefficients of variation are <8% and <10%, respectively.³

Statistics

A power calculation was performed based on previously reported data, which established a normal TC concentration of 174 ± 58 mg/dL in 33 healthy dogs, and a TC concentration of 360 ± 147 mg/dL in 11 dogs with DM.⁴ Using these values and assuming a normal distribution of TC concentration within each study group, 8 dogs would be required in each group to detect a statistically significant difference in cholesterol concentration between the groups, with a power of 0.8 and type I error rate of 0.05. However, additional samples from eligible dogs were analyzed as they became available until the end of the allocated research time.

Some lipoprotein fraction concentrations were not normally distributed as determined by the Shapiro-Wilk test, so the nonparametric Mann-Whitney test was used for comparison of median lipoprotein concentrations in dogs with DM compared to healthy dogs. The Mann-Whitney test was also used to determine if there was a significant difference in the age, weight, or BCS of dogs with and without DM. Fisher’s exact test was used to compare the sex distribution and BCS in dogs with and without DM. Spearman’s correlation was used to assess if there was an association between ApoB concentration or BCS with TC, LDL-C, HDL-C, TG, VLDL-C, or non-HDL-C concentrations in all dogs. For the Spearman’s correlation tests, dogs with and without DM were analyzed together. A P-value <0.05 was considered significant. All statistical analyses were performed by a statistical software package.⁵

Results

Twenty-two dogs with DM and 9 healthy dogs were enrolled. The median (range) age of dogs with DM and of healthy dogs was 9 years (4–12 years) and 9 years (8–13 years), respectively. Thirteen neutered males and 9 neutered females were included in the group of dogs with DM, whereas 5 neutered males and 4 neutered females were included in the healthy group of dogs. No significant difference in age or sex distribution was found between groups. The most common breed in each group was mixed breed with 6 mixed breed dogs in the DM group and 5 mixed breed dogs in the healthy group. The DM group also included Yorkshire Terriers (2), Jack Russell Terriers (2), Cocker Spaniels (2), and 1 each of West Highland White Terrier, Miniature Pincher, Polish Lowland, Cairn Terrier, Shih Tzu, Labrador Retriever, Pug, Bichon, Pomeranian, and Doberman Pincher. The remainder of the healthy group included 1 each of Dachshund, Siberian Husky, Boston Terrier, and Cairn Terrier. Median body weight of dogs with and without DM was 8.6 kg (4.8–34.2 kg) and 20.2 kg (6.3–30.4 kg), respectively. Median ApoB concentration of dogs with and without DM was 5 (2–8) and 5 (4.5–6.6), respectively. There was no significant difference between dogs with and without DM, in regard to weight or BCS. BCS was also not significantly different in females compared to males, when all dogs with and without DM were analyzed as 1 group. The associations between BCS and each of the lipoprotein fractions were also not statistically significant when all dogs with and without DM were analyzed as 1 group.

Thirteen of 22 dogs in the DM group were fed dry or canned Hill’s W/D as the main portion of their diet. One dog received Royal Canin Gastrointestinal Low Fat, and 1 dog each was fed Royal Canin HP, Royal Canin Glycobalance, or Purina OM. Four other dogs with DM received mixtures of various over-the-counter foods and home-prepared food. Healthy dogs were fed various over-the-counter adult maintenance formulas. Eighteen of 22 dogs (82%) with DM were treated with neutral protamine Hagedorn insulin; 2 dogs (9%) were treated with Lente insulin, and 2 other dogs (9%) were treated with glargine insulin at the time of enrollment. Insulin doses ranged from 0.12 to 1.03 U/kg given subcutaneously twice daily.

All plasma lipid fraction concentrations (TC, TG, VLDL-C, LDL-C, non-HDL-C, HDL-C, and ApoB) were significantly higher in dogs with DM compared to healthy dogs. Median concentrations of lipid fractions in dogs with and without DM and associated p-values are listed in Table 1. The greatest difference was detected in LDL-C concentration, which was 4.2 times higher in dogs with DM compared to healthy dogs. Non-HDL-C, VLDL-C, TG, and TC concentrations were 3.7, 2.2, 2.1, and 1.7 times higher, respectively, in dogs with DM compared to healthy dogs. The smallest, yet still significant, differences were detected in HDL-C and ApoB concentrations, which were each 1.2 times higher in dogs with DM compared to controls. Ten (45%) of 22 dogs with DM had either non-HDL-C concentration greater than 130 mg/dL or LDL-C concentration greater than 100 mg/dL. Nine of these

Table 1. Median (range) concentration of lipoprotein lipids in dogs with and without diabetes mellitus (DM) expressed in mg/dL. * A P-value is reported for the comparison of each median lipid fraction in dogs with and without DM.

|        | TC   | VLDL-C | LDL-C | HDL-C | TG   | Non-HDL-C | ApoB |
|--------|------|--------|-------|-------|------|-----------|------|
| Dogs with DM (22) | 343.5 (171–515) | 27 (7–250) | 68 (4–248) | 200 (83–285) | 122 (25–2310) | 114 (56–323) | 320 (166–780) |
| Dogs without DM (9) | 197 (105–267) | 12 (1–24) | 16 (4–45) | 168 (100–204) | 57 (42–486) | 31 (5–63) | 258 (106–379) |
| P-value | <0.001 | 0.011 | <0.001 | 0.029 | 0.0079 | <0.001 | 0.025 |

*TC, total cholesterol; VLDL-C, very low-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; non-HDL-C, non-high-density lipoprotein cholesterol; ApoB, apolipoprotein B.
10 dogs (90%) had non-HDL-C concentration greater than 130 mg/dL, and 5 dogs (50%) had LDL-C concentration greater than 100 mg/dL. Four of the 5 dogs with LDL-C concentration greater than 100 mg/dL also had non-HDL-C concentration greater than 130 mg/dL. In 21 of 22 dogs with DM (95%), at least 1 of the lipid fraction concentrations was increased above the highest concentration noted for that specific lipid fraction in healthy dogs. A significant association was found between ApoB and LDL-C concentrations (Spearman’s rho = 0.41, P-value = 0.022) and between ApoB and non-HDL-C concentrations (Spearman’s rho = 0.40, P-value = 0.027). The associations between ApoB and other lipoprotein fractions were not statistically significant. No significant differences were detected in any of the lipid fractions between males and females, when data were analyzed separately for dogs with DM and dogs without DM, and when data from all dogs with and without DM were analyzed together.

Median blood glucose concentration measured in 22 dogs with DM, every 2 hours over 8–12 hours after insulin administration and feeding was 170 mg/dL (67–557 mg/dL) and was significantly higher than median fasted blood glucose concentration measured at the time of enrollment in 9 healthy dogs (88 mg/dL, range 84–115 mg/dL, P-value = 0.0031).

**Discussion**

The results of our study demonstrate that in dogs with DM, dyslipidemia is characterized by pronounced increases in LDL-C and non-HDL-C concentrations, although VLDL-C, TG, TC HDL-C, and ApoB concentrations are also increased. In dogs with DM, the lipid fraction most increased in magnitude is LDL-C. However, the lipid fraction that was increased in the greatest number of dogs was non-HDL-C. It is therefore concluded that increases in both LDL-C and non-HDL-C are major causative predictors of cardiovascular disease in humans and the goal of lipid-lowering treatment in humans with type 1 or type 2 DM is to decrease LDL-C and non-HDL-C to less than 100 mg/dL and 130 mg/dL, respectively.20,21 Although ApoB concentration was significantly higher in dogs with DM compared to healthy dogs, the magnitude of increase was relatively small compared to the increase observed in other lipid fractions. Furthermore, ApoB concentration was significantly associated with LDL-C and non-HDL-C concentrations. It is therefore concluded that ApoB might not be useful for quantification of dyslipidemia in dogs with DM, especially if LDL-C and non-HDL-C concentrations are known.

Control dogs were selected to be of a similar age to dogs with DM because age influences lipid profiles.3 All control dogs were neutered, as were dogs with DM. This neuter status is representative of the general hospital population and is especially true for female dogs with DM, in which neutering is recommended at the time of DM diagnosis. Control dogs were selected to be of similar age and neuter status and to increase the likelihood that differences detected in lipid fractions between dogs with and without DM were attributed to presence or absence of disease rather than to age or neuter status.

This study has several limitations, one of which is that dogs with DM had concurrent disorders that may have influenced treatment with medications other than insulin. Some of the concurrent disorders and medications could have influenced the concentration of lipoprotein fractions. Similarly, although most dogs with DM were fed a low fat diet, the diet was not standardized and dogs were fed a variety of diets. Although all lipid samples were obtained after a 12-hour fast (some dogs were on oral medications), diet and medications could still have influenced the study findings. The number of hours dogs were fasted before blood sampling for the purpose of lipid analysis was also variable and ranged from 8 to 12 hours. The fast duration depended on the number of hours that the dog was in the hospital, after eating and insulin administration. The results of this study investigating the effect of lipid-lowering medications in dogs with DM.

LDL is composed of a hydrophobic cholesteryl ester-rich core, which is surrounded by phospholipids and 1 large ApoB molecule. ApoB facilitates blood-borne transportation of lipids, and the binding of LDL to receptors at the target tissues. LDL contains mainly cholesterol, and although it is also comprised of some TG, it is not rich in TG. In contrast to LDL-C, non-HDL-C concentration is the sum of cholesterol, TG-rich lipoproteins (chylomicrons, chylomicron remnants, intermediate density lipoprotein, and VLDL) and LDL-C. In this study, a greater number of dogs had increased non-HDL-C concentration compared to the number of dogs that had increased LDL-C concentration. It is therefore concluded that TG-rich lipoproteins have an important role in the dyslipidemia of dogs with DM.

ApoB was measured because it provides an estimation of the number of LDL particles and is an important predictor of cardiovascular disease in humans.8,9 ApoB is used to estimate the number of small, dense LDL particles, which are thought to be more atherogenic than large LDL particles. Although ApoB concentration was significantly higher in dogs with DM compared to healthy dogs, the magnitude of increase was relatively small compared to the increase observed in other lipid fractions. Furthermore, ApoB concentration was significantly associated with LDL-C and non-HDL-C concentrations. It is therefore concluded that ApoB might not be useful for quantification of dyslipidemia in dogs with DM, especially if LDL-C and non-HDL-C concentrations are known.

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therefore represent findings from the real and diverse clinical population of dogs with DM fed different diets, and with a variety of concurrent disorders. Presence of concurrent disorders is common in dogs with DM, possibly due to their age. However, metabolic and systemic derangements associated with DM, such as hyperglycemia, hyperlipidemia, or hypertension, can also increase the risk of concurrent disorders. Had the study focused on dogs with DM only, the generalizability of the findings to the true population of dogs with DM would have been limited. The decision to include dogs with concurrent disorders in the study was taken to ensure that the study findings were relevant to the clinical population of dogs with DM.

Another study limitation is that dogs with various degrees of glycemic regulation were included. Further studies focusing on subgroups of dogs with DM will be needed to determine if the concentrations of lipoprotein fractions in dogs with poorly regulated DM are different than in well-regulated DM, and the extent to which concurrent disorders influence lipoprotein lipid profiles in dogs with DM.

Several methods for lipoprotein quantification have been reported in dogs, with issues such as ease of use, equipment availability, time constraints, and accuracy being considered. A manual and automated lipoprotein electrophoresis, density-gradient ultracentrifugation, various high-performance liquid chromatography methods, and chemistry analyzers have been used to quantify lipoproteins in dogs. There is currently no established consensus on the gold standard methodology for lipoprotein measurements in dogs. Therefore, it is possible that lipoprotein concentrations measured by a different methodology, such as manual lipoprotein electrophoresis, could yield somewhat different results than those reported in this study. However, the goal of this study was to compare lipoprotein lipid concentrations in dogs with and without DM. The same methodology was used in diabetic and control dogs, and any imprecision in lipoprotein quantification would have affected both groups. Furthermore, ApoB was significantly increased in dogs with DM, the magnitude of increase was not large. ApoB is also significantly associated with LDL-C and non-HDL-C concentrations. Abnormally high LDL-C and non-HDL-C increase the risk of cardiovascular disease in humans, and statins are used to lower them in humans with type 1 and type 2 DM. Future studies could investigate the clinical utility of statins in dogs with DM. Although ApoB was significantly increased in dogs with DM, the magnitude of increase was not large. ApoB is also significantly associated with LDL-C and non-HDL-C concentrations. Therefore, ApoB will not necessarily be incorporated into future studies of dyslipidemia in dogs with DM.

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**Footnotes**

a Cell-Dyn 3700; Abbott Laboratories, Abbott Park, IL
b Vitros Chemistry System 350, Ortho-Clinical Diagnostics, Rochester, NY
c Accu-Chek Performa, Roche Diagnostics Corp, Indianapolis, IN
f COBAS c311 analyzer, Roche Diagnostics, Indianapolis, IN
k Canine Glycobalance, Royal canin, St. Charles, MO
m Canine Hydrolyzed Protein Adult HP, Royal canin, St. Charles, MO
n OM Overweight Management™ Canine Formula, Purina, St. Louis, Missouri
o Humulin N, Eli Lilly and Co, Indianapolis, IN
p Porcine insulin zinc suspension, Merck Animal Health, Summit, NJ
q Lantus, Sanofi-Aventis US LLC, Bridgewater, NJ
r Canine Gastrointestinal Low Fat, Royal canin, St. Charles, MO
s W/D diet, Hill's Pet Nutrition Inc, Topeka, KS

d Vacuette 4 ml K3E EDTA K3, Greiner bio-one, Monroe, NC

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Conflict of Interest Declaration: The authors declare no conflict of interest.

Off-label Antimicrobial Declaration: The authors declare no off-label use of antimicrobials.

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