Regression formula to predict the International Federation of Clinical Chemistry and Laboratory Medicine measure of alkaline phosphatase activity in canine blood based on the Japan Society of Clinical Chemistry reference method

Akihisa HATA1,2), Noboru FUJITANI1,2)*, Chie TANAKA2), Noriko MATSUDA2), Michiko TAKAISHI2), Takako SHIMOKAWA MIYAMA1) and Fumio HOSHI1)

1)Faculty of Veterinary Medicine, Okayama University of Science, Ikoino-oka 1-3, Imabari, Ehime 794-8555, Japan
2)Biomedical Science Examination and Research Center, Okayama University of Science, Ikoino-oka 1-3, Imabari, Ehime 794-8555, Japan

ABSTRACT. The Japan Society of Clinical Chemistry reference method (JSCC method) is used to measure alkaline phosphatase (ALP) activity only in Japan. Other countries use the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) reference method to measure ALP activity. Since April 2020, human medical institutions in Japan have been gradually switching to the IFCC method. However, it is unclear whether the supply of reagents required for the JSCC method will be steady in the future. Additionally, the comparison of the performances and accuracies of these two methods for measuring ALP values remains uncertain in several animal species. In this investigation, we measured canine ALP activity using both methods and developed a formula to interconvert the two resulting values. The regression formula for ALP values measured using the modified JSCC (x) and IFCC (y) methods was determined as log10 y=0.960 log10 x−0.395 (r=0.997). However, the correlation between values based on JSCC and IFCC methods can change depending on the composition of ALP isozymes. Therefore, the developed formula can currently serve as a provisional strategy in calculating ALP levels. Nevertheless, this formula might avoid confusion in the clinical field during the transition from the JSCC to the IFCC method when both measurement values co-exist.

KEY WORDS: canine alkaline phosphatase, International Federation of Clinical Chemistry and Laboratory Medicine, isozyme, Japan Society of Clinical Chemistry

Elevated alkaline phosphatase (ALP) activity levels in mammalian blood are commonly associated with diseases such as primary liver disease, drug-induced liver injury, pancreatitis, hypercortisolism, and metabolic bone disease [1]. In veterinary medicine, blood biochemistry examinations, including the measurement of ALP activity, are generally conducted using medical analyzers and reagents developed for evaluating human samples. The Japan Society of Clinical Chemistry (JSCC) reference method is used for ALP measurements in veterinary medicine only in Japan. All other countries use the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) reference method to measure ALP activity. However, since April 2020, medical institutions in Japan began gradually switching to the IFCC method. In addition, it is unclear whether the supply of reagents to measure ALP activity with the JSCC method will continue in the future.

The JSCC method differs from the IFCC method in terms of buffer solution composition. The former uses 2-ethylaminoethanol, whereas the latter uses 2-amino-2-methyl-1-propanol [3, 5]. This difference in buffer solution results in a considerable variation in measured ALP values [3]. The relationship between the JSCC and IFCC methods in measuring ALP values remains unclear in several animal species. Hence, values measured using these two methods cannot be used interchangeably. We examined the relationship between canine ALP values determined using JSCC and IFCC methods and generated a formula to convert and compare the values obtained using each method.
MATERIALS AND METHODS

Sample and data collection
A total of 113 residual canine blood samples from blood biochemistry examinations conducted at the Okayama University of Science Veterinary Teaching Hospital (Imabari, Ehime, Japan) were collected. We ensured not to analyze samples from the same dog twice.

The numbers of male and female dogs were 57 and 56, respectively. The breeds of dogs and specimen numbers were as follows: 20 Toy Poodles, 20 Miniature Dachshunds, 11 mixed breed dogs, 8 Shiba Inus, 7 Chihuahuas, 5 Welsh Corgis, 5 Shih Tzus, 4 Golden Retrievers, 4 Papillons, 4 French Bulldogs, 4 Miniature Schnauzers, and 21 other breeds. A history of any present illness that may increase the blood ALP concentration included: 10 cases of hepatobiliary disease, 6 cases of bone disease, and 2 cases of kidney disease. One specimen had bone and hepatobiliary disease. The average age of the dogs was 8.7 ± 4.2 years, whereas 5 dogs were less than 1 year of age.

Lithium heparin was used as an anticoagulant. The blood was centrifuged at 1,500 × g at room temperature for 10 min immediately after sampling. The plasma was collected in a cryovial and frozen at −30°C until further analysis.

Analysis of plasma ALP activity
Unless otherwise specified, reagents were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). ALP activity was measured using the JSCC method by employing ALP II-J2, which consists of 2-ethylaminoethanol buffer solution and 4-nitrophenyl phosphate-substrate solution; the lower and upper limits of quantitation using ALP II-J2 are 1.5 U/l and 2,000 U/l, respectively. ALP activity measured using the IFCC method employed ALP IFCC, which consists of 2-amino-2-methyl-1-propanol buffer solution and 4-nitrophenyl phosphate-substrate solution; this analytical reagent, the lower limit of quantitation is 1 U/l, and the upper limit is 700 U/l. During preliminary analyses, the dilution linearity in the measurable range of both methods was determined using canine blood containing high ALP concentrations (Fig. 1). The enzyme calibrator Wako was used for calibration. Control Wako-I and Wako-II were used for quality control. As calculated using the JSCC method, the assigned ALP values of Control Wako-I and Control Wako-II were 202 U/l and 576 U/l, respectively. As calculated using the IFCC method, the assigned ALP value of Control Wako-I was 76 U/l, and that of Control Wako-II was 250 U/l. A Hitachi 3100 clinical analyzer (Hitachi High-Technologies Corp., Tokyo, Japan) was used for ALP analysis; the reaction parameters for ALP analysis were set according to the manufacturer’s recommendations. All incubations were performed at 37°C. In the JSCC method, 2.5 µl of plasma and 160 µl of 2-ethylaminoethanol buffer solution were pipetted into the reaction cuvette and incubated for 5 min. Then, 40 µl of 4-nitrophenyl phosphate-substrate solution was added and incubated for 1 min; the change in absorbance was monitored at 405 nm for 4 min. In the IFCC method, 4 µl of plasma and 160 µl of 2-amino-2-methyl-1-propanol buffer solution were pipetted into the reaction cuvette and incubated for 5 min. Then, 40 µl of 4-nitrophenyl phosphate-substrate solution was added and incubated for 1.5 min; the change in absorbance was monitored at 405 nm for 3.5 min. For the JSCC method, the inter-assay coefficient of variation (CV) was 0.82%, and the intra-assay CV was 0.79% using Control Wako-II. For the IFCC method, the inter-assay CV was 0.68%, and the intra-assay CV was 0.74% using Control Wako-II.

ALP isozyme analysis
Unless otherwise specified, reagents were purchased from Helena (Saitama, Japan). The percentage of each ALP isozyme was analyzed using agarose gel electrophoresis using the Quick Gel ALP system. Plasma samples were treated with neuraminidase (ALP separator). The electrophoretic conditions were as follows: gel temperature, 20°C; voltage, 250 V; and run time, 20 min. ALP isozyme bands were detected using 3-indoxyl phosphate disodium salt as the ALP substrate, and nitro blue tetrazolium as the dye. The dried agarose gel was scanned using a flat head scanner (EPSON GT-F740, Tokyo, Japan), and the percentage of each ALP isozyme was calculated using ImageJ software (version 1.52o April 23, 2019, National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/). A gel ALP control was used for quality control. Generally, liver ALP (LALP), bone ALP (BALP), and corticosteroid-induced ALP (CALP) are detected in dog plasma [2]. Each ALP isozyme was identified by assessing the mobility of bands and inhibiting LALP and BALP with levamisole (levamisole hydrochloride, Nacalai Tesque, Kyoto, Japan) [6].

Statistical analysis
Regression analysis between the JSCC and IFCC method values was performed using log10-transformed values. ALP values obtained using both methods were plotted on a scatter diagram, with the x-axis representing the values obtained using the JSCC method and y-axis representing those obtained using the IFCC method. The regression formula was developed using the major
axis regression method in Validation-Support/Excel Ver.3.5 (JSCC, Quality Management Expert Committee). The 95% confidence interval was calculated via the bootstrap method.

The statistical significance of differences across groups was calculated using the Kruskal-Wallis test. Differences with a *P*-value of less than 0.05 were considered statistically significant. SPSS (version 19, SPSS, Inc., Chicago, IL, USA) was used for statistical analyses.

The residuals of each regression formula were calculated as follows: residual=(actual measurement value using the IFCC method)−(IFCC value estimated using regression formula). The standardized residuals of each regression formula were calculated as follows: standardized residual=(residual)/(residual standard deviation).

**RESULTS**

**ALP activity in the samples**

Among the 113 samples, the median values (interquartile range) of the JSCC and IFCC methods were 241 (142–532) U/l and 82 (46–164) U/l, respectively. In five dogs under 1 year of age, the median values obtained using the JSCC and IFCC methods were 312 (228–369) U/l and 105 (76–125) U/l, respectively.

The difference in ALP activities, calculated with both the JSCC and IFCC methods, across canine breeds, was analyzed using the Kruskal-Wallis test. The *P*-values for the inter-species ALP values obtained with the JSCC and IFCC methods were 0.652 and 0.711, respectively. Thus, the differences in ALP activity were not significant among canine breeds.

**Regression between the JSCC and IFCC methods**

Figure 2 shows the scatter plots of ALP values. For the 113 samples assessed, the regression formula was log_{10} y=0.960 log_{10} x−0.395 and the correlation coefficient (r) was 0.997. The upper and lower limits of the 95% confidence interval were represented by log_{10} y=0.977 log_{10} x−0.353 and log_{10} y=0.942 log_{10} x−0.438, respectively (Fig. 2A). As a consequence, canine blood ALP values determined using the IFCC method were approximately one-third of those determined using the JSCC method.

The standardized residual plots drawn using the regression equation are shown in Fig. 2B. In these plots, the residual values were equally distributed, although variation in the high-concentration specimens was larger than that in the low- and medium-concentration specimens (Fig. 2B). To analyze the source of this variation, the specimens were divided into four groups after sorting the ALP concentrations in ascending order according to the JSCC method results: quartile 1 (Q1), 15–142 U/l; Q2, 144–241 U/l; Q3, 253–532 U/l; and Q4, 535–1,615 U/l.

The regression formulas for groups of dogs under 1-year-old, with bone disease, and with hepatobiliary disease, are shown in Table 1. The slope of the under 1-year-old group was larger than that of the 1-year-old or older group. The slope of the bone disease group was larger than that expected for the hepatobiliary and other disease groups. The slope of the hepatobiliary disease group was slightly smaller than that expected for the bone and other disease groups.

![Fig. 2. Correlation between alkaline phosphatase (ALP) activities estimated using the Japan Society of Clinical Chemistry (JSCC) and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) reference methods in 113 canine blood samples. The x-axis represents the values obtained using the JSCC method and y-axis represents those obtained using the IFCC method. Regression analysis between the values based on the JSCC and IFCC reference methods was performed using log_{10} transformed values. (A) Regression analysis comparing the JSCC and IFCC values of all specimens represented by the following equation: log_{10} y=0.960 log_{10} x−0.395. (B) Standardized residual plots from the regression formulas of all specimens.](image-url)
Composition of ALP isozymes in canine blood

Of the ALP isozymes analyzed in 113 canine blood samples, four were below the lower limit of detection. Of the remaining 109 samples, the median values (interquartile range) of BALP, LALP, and CALP were 17.9% (10.2–29.8), 70.9% (64.2–77.7), and 7.7% (4.3–12.6), respectively.

The percentages of isozymes in Q1–4 are shown in Fig. 3. The percentage of isozymes (BALP, LALP, and CALP) between Q1–4 was analyzed using the Kruskal-Wallis test. BALP and CALP showed a significant difference among the ALP concentration groups (BALP, \( P < 0.001 \); LALP, \( P = 0.463 \); and CALP, \( P < 0.001 \)). The percentage of BALP overall tended to be lower in the group with the highest ALP concentration (Q4) than in the other groups. In contrast, the percentage of CALP was higher in Q4 than in the other quartiles.

The isozyme composition in the groups comprising under 1-year-old dogs, dogs with bone disease, and those with hepatobiliary disease is shown in Table 1. BALP activity in the under 1-year-old group was higher than that in the 1-year-old or older group. In contrast, LALP activity in the under 1-year-old group was lower than that in the 1-year-old or older group. BALP in the bone disease group was higher than that expected for the hepatobiliary and other disease groups. Statistical analyses were not performed, as the sample sizes differed among groups.

Differences in the isozyme composition across canine breeds were analyzed using the Kruskal-Wallis test, but no significant differences were detected (BALP, \( P = 0.296 \); LALP, \( P = 0.424 \); CALP, \( P = 0.215 \)).

DISCUSSION

Our results showed that canine blood ALP values analyzed using the IFCC method were approximately one-third of those determined using the JSCC method. The provisional regression formula comparing the JSCC (x) and IFCC methods (y) for human blood ALP has been reported as \( y = 0.357 \log_{10} x - 1.103 \) [7]. In humans, differences in ALP activities determined via the JSCC and IFCC methods occur because of differences in the composition of the buffer solution used. The activity of every human ALP isozyme analyzed using the IFCC method was found to be lower than that measured using the JCSS method [3]. Similar differences in enzymatic activities are likely observed for canine ALP isozymes.

Based on standardized residual plots of all specimens, we observed specimens whose ALP activity values were remarkably higher or lower than expected (Fig. 2B). It is important to note that it is more difficult to reasonably estimate and accurately compare specimens with a high-ALP activity than those with a low- or medium-ALP activity. The difference in the ALP isozyme composition likely caused the unexpectedly high or low values along the x-axis and the deviation between measured IFCC and expected IFCC values in high-ALP specimens.

BALP and CALP showed significant differences across the ALP concentration groups (Fig. 3). The percentage of BALP was lower...
in the group with the highest ALP concentration (Q4) than in the other groups, whereas the percentage of CALP was higher in Q4 than in the other groups.

Under the same measurement conditions, the activities of different ALP isozymes may vary. Further, changing the chemical composition of the reagent buffer can result in a non-uniform change in the activity of each ALP isozyme [3]. For example, when human ALP is analyzed using the JSCC method, the activity of intestinal ALP is higher than that of other ALP isozymes. In contrast, using the IFCC method, the activity of intestinal ALP is lower than that of other isozymes [3]. Canine CALP is a transcriptional product of the intestinal ALP isozyme-encoding gene [4]. The specific activity of canine ALP isozymes in each buffer solution was not investigated. However, the behavior of CALP may be similar to that of human intestinal ALP as measured with both the JSCC and IFCC methods. As evidenced in scatter plots showing JSCC- and IFCC-based measurements performed in humans, intestinal ALP activity, which is detected in B and O blood group samples, and placental ALP, which is detected in pregnant women, diverges from the regression line [7].

Our results and those of previous studies indicate that the ALP isozyme composition influences the slope of the regression formula between JSCC- and IFCC-based measurements of canine blood ALP.

Canine blood ALP values analyzed using the IFCC method were approximately one-third of those determined via the JSCC method. The regression formula for canine ALP values to compare the JSCC (x) and IFCC (y) methods was found to be \( \log_{10} y = 0.960 \log_{10} x - 0.395 \). However, this relationship can change depending on the ALP isozyme composition. We consider this formula a provisional strategy for estimation. Of note, there was a large variation in the standardized residual plots of high-concentration ALP specimens (Fig. 2B). As a consequence, the suitability of the regression formula for high-concentration specimens is lower than that for low- and medium-concentration specimens.

Furthermore, it is necessary to determine regression formulas for ALP values measured using the JSCC and IFCC methods in other animal species.

The present study had some limitations. First, this was not a multicenter study. Because the study specimens were residual blood samples from dogs admitted to a secondary hospital, there may have been sampling bias. Second, the study was conducted using frozen plasma specimens. The degradation of ALP and/or changes in ALP activity may have occurred during frozen storage. Finally, we used reagents from one company (Fujifilm Wako Pure Chemical) for both the JSCC and IFCC methods. Although both reagents are standardized, there may have been a slight difference in reactivity compared with those from other companies.

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Fig. 3. Distribution of alkaline phosphatase (ALP) isoenzymes in each quadrant of ALP concentration.
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