Changes in the blood lactate dehydrogenase measurements in canines and felines following the international standardization of the assay method

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ABSTRACT. Lactate dehydrogenase (LDH) in blood is measured using the Japanese Society of Clinical Chemistry (JSCC) method in Japan and the International Federation of Clinical Chemistry (IFCC) method in other countries. In human clinical practice, the IFCC method replaced the JSCC method due to international standardization. Moreover, veterinary LDH measurement will also eventually shift to the IFCC method. However, the relationship between the IFCC and JSCC methods for LDH in various animals and whether they can be equated or not have not yet been investigated. This study aimed to present the changes in measurements in canines and felines after switching to the IFCC method. The plasma LDH activity of canines (N=177) and felines (N=115), who visited a secondary care veterinary clinic, was measured using the JSCC and IFCC methods. The IFCC/JSCC ratio was <1.0 in 85% of canines and 88% of felines, indicating that the IFCC method tended to show lower values than the JSCC method, presumably because LDH5 is dominant among the LDH isozymes in canines and felines. The increase in the systematic errors of both assays was in the high value range, with some samples exceeding the error tolerance from near the upper end of the reference range. When switching to the IFCC method for LDH measurement in canines and felines, each institution should consider whether the reference range and clinical diagnostic values established by the JSCC method are appropriate for continued use.

KEYWORDS: canine, feline, International Federation of Clinical Chemistry and Laboratory Medicine, Japan Society of Clinical Chemistry, lactate dehydrogenase

Lactate dehydrogenase (LDH; EC 1.1.1.27) is an enzyme that catalyzes the reaction—Lactic acid + nicotinamide adenine dinucleotide (NAD\(^+\)) ↔ Pyruvate + dihydronicotinamide adenine dinucleotide (NADH) + H\(^+\)—in the final step of the glycolytic system. Moreover, it is widely present in animals, plants, and microorganisms [1, 9].

LDH is a tetramer composed of two monomers, H (cardiac muscle type) and M (skeletal muscle type), which has five isozymes (LDH1 to LDH5) depending on the combination of the two monomers [9]. Blood LDH is an absconding enzyme, and its increase indicates the presence of tissue damage. In general, elevated blood LDH in mammals is observed in diseases such as hematologic, myocardial, muscular and liver diseases, and various malignant tumors [2, 6]. The ratio of each LDH isozyme differs from tissue to tissue, and isozyme analysis is used to estimate the site of damage [10, 11].

Clinical laboratories in veterinary medicine, particularly in biochemical testing, often use medical analyzers and measuring reagents developed for human use. The same is true for LDH activity assay and clinical reagents based on the Japanese Society of Clinical Chemistry reference method (JSCC method) in Japan and International Federation of Clinical Chemistry reference method (IFCC method) in other countries, which have been used as assay reagents.

In clinical practice, the Japanese LDH and alkaline phosphatase (ALP) measurement method shifted to the IFCC method last April 1, 2020 for the international standardization of clinical laboratories. Prior to this, the relationship between both IFCC and JSCC measurements was clarified to avoid confusion during the transition period. The difference between the two measurements of LDH in human blood was minor within the reference range; thus, there was no need for conversion, and conventional values could be used for the reference range as well [8]. The relationship between the IFCC and JSCC method measurements has been reported for ALP in dogs, cats, and dairy cattle and for LDH in dairy cattle [4, 5, 14]. However, it has not been clarified for other animal species.

Whether the supply of reagents for the conventional JSCC method will continue remains unclear. Moreover, veterinary LDH
measurement will eventually have to switch to the IFCC method. This study aimed to present the changes in measurements in canines and felines after switching to the IFCC method. For this purpose, an investigation of the relationship between both the JSCC and IFCC measurements was conducted on blood samples from canines and felines that visited secondary veterinary clinics. In addition, LDH isozyme analysis was performed because LDH isozyme patterns may be involved in the discrepancy between the IFCC and JSCC measurements [7, 8].

MATERIALS AND METHODS

Ethical approval

Residual blood from the biochemical tests of canines and felines were used. Canines and felines were not subject to ethical considerations according to the Okayama University of Science ethics committee on clinical investigation (examination number 2020-0007) because blood was not sampled from the animals in this study.

Sample and data collection

Individual information was completely anonymized, and measurements were completely separated from the personal information. A total of 177 canine and 115 feline residual blood samples from blood biochemistry analyses at the Biomedical Science Examination and Research Center, Okayama University of Science (Imabari, Ehime, Japan) were collected.

Lithium heparin was used as an anticoagulant for canine and feline blood. Blood samples were centrifuged (1,500 × g, 10 min), wherein plasma was separated. Subsequently, they were frozen at −80°C until further analysis.

The median age of the canines in the study was 8 years (range 0–16), the ratio of females to males was 44:56, and the median weight was 6 kg (range 2–39). There were 36 dog breeds, for which plasma was used, and the top 10 breeds were as follows: Toy Poodle (N=29), Miscellaneous (25), Miniature Dachshund (21), Shiba Inu (13), Welsh Corgi Pembroke (8), Chihuahua (8), Miniature Schnauzer (7), Shetland Sheepdog (6), French Bulldog (6), and Jack Russell Terrier (4).

The median age of the cats, whose plasma was used, was 8 years (range 0–17), the ratio of females to males was 45:55, and the median weight was 4 kg (range 1–10). There were 10 cat breeds with the following numbers of cats each: mongrel (N=70), Russian Blue (13), American Shorthair (7), Scottish Fold (5), Somali (4), Norwegian Forest Cat (4), Ragdoll (2), Bengal (1), Munchkin (1), and Maine Coon (1).

Analysis of blood LDH activity

LDH activities were measured using the JSCC and IFCC methods. Reagents were obtained from Fujifilm Wako Pure Chemical (Osaka, Japan) unless specified otherwise. The JSCC method for LDH analysis employed the LD-J, which consists of L-lactate in diethanolamine buffer solution and β-nicotinamide adenine dinucleotide solution. The lower limit of quantitation was 5.4 U/L, and the upper limit was 1,300 U/L. The IFCC method for LDH measurement employed the LD-IF, which consists of L-lithium lactate in N-methyl-D-glucamine buffer solution and β-nicotinamide adenine dinucleotide solution. The lower limit of quantitation was 5 U/L, and the upper limit was 1,300 U/L.

A Hitachi 3100 clinical analyzer (Hitachi High-Technologies Corp., Tokyo, Japan) was used for LDH analyses. The enzyme calibrator, Wako, was used for calibration. Control Wako-I and Wako-II were used for quality control.

Analysis of LDH isozyme

LDH isozymes were analyzed using agarose-gel electrophoresis. All reagents and equipment were obtained from Helena Laboratories, Inc. (Saitama, Japan). Agarose-gel electrophoresis was performed using an LDH isozyme analysis kit (Quick gel LD (QG)) according to the manufacturer’s instructions. The CK/LD control was used as a control. The percentage of LDH isozymes was calculated by measuring the formazan produced from the following reaction system with a densitometer:

\[
\text{L-Lactate} + \text{NAD}^{+} + \text{Pyruvic acid} + \text{NADH} + \text{H}^{+}
\]

\[
\rightarrow \text{NAD}^{+} + \text{Diaphorase} (\text{FDA})
\]

\[
\text{Diaphorase} (\text{FDA}) + \text{Nitro blue tetrazolium chloride} \rightarrow \text{Diaphorase (FDA) + Formazan + H}^{+}
\]

Electrophoresis and densitometric analysis were performed using an Automated System for Gel Electrophoresis Epalyzer 2 Junior.

Statistical analysis

LDH values obtained using both the methods were plotted using a scatter diagram, wherein the x- and y-axes represented the values obtained using the JSCC and IFCC method, respectively. Regression formulas were developed using standard major axis regression in Validation-Support/Excel Ver.3.5 (JSCC, Quality Management Expert Committee), and 95% confidence interval (CI) was calculated via bootstrapping.

A Brand–Altman plot was prepared, wherein x-axis is the mean value of the JSCC and IFCC measurements and y-axis is the difference between the two measurements (IFCC measurement–JSCC measurement), to evaluate the agreement of LDH measurements for both methods and the presence of systematic errors. The upper and lower limits of the acceptable range of error for both measurements were calculated as the mean ± 1.96 standard deviation (SD) of the error.

Spearman’s rank correlation coefficients were determined between the IFCC/JSCC ratio and age, weight, LDH activity of the IFCC method, LDH of the JSCC method, LDH 1%, LDH 2%, LDH 3%, LDH 4%, and LDH 5% to explore factors affecting the relationship between IFCC and JSCC measurements.
Comparisons between the groups were made using the Kruskal–Wallis test to determine whether the distribution of the IFCC/JSCC ratio differed by dog and cat breeds, and sex. Correlation analysis and between-group comparisons were performed using IBM SPSS Statistics for Windows, version 19 (IBM Corp., Armonk, NY, USA). Statistical significance was set at \( P<0.05 \).

**Reference ranges for LDH activity and LDH isozymes**

Since the JSCC method is used only in Japan, the validity of using LDH reference ranges measured in other countries has not been confirmed.

The results of the LDH isozyme analysis method using electrophoresis vary depending on the gel material used and other factors such as the conditions of the gel electrophoresis. Therefore, it is possible to compare the results only with data that used the same method. Therefore, in this study, the LDH activity values of the JSCC method used data investigated by FUJIFILM VET Systems Co., Ltd. (Tokyo, Japan), a major clinical testing company in Japan. Plasma LDH reference range is set at \( \leq 109 \) U/L for canines and \( \leq 187 \) U/L for felines. For the LDH isozyme analysis method, data presented by FUJIFILM VET Systems Co., Ltd., which uses the agarose-gel electrophoresis method as in this study, were also used [3]. The reference ranges for the canines LDH isozyme fraction by FUJIFILM VET Systems Co., Ltd. are as follows; LDH1, 1.1–22.6%; LDH2, 0.8–10.6%; LDH3, 2.1–31.4%; LDH4, 9.7–21.2%; and LDH5, 21.3–83.8%. The reference ranges for felines are as follows; LDH1, 0–21.6%; LDH2, 1.5–22.0%; LDH3, 8.0–21.5%; LDH4, 15.5–29.7%; and LDH5, 20.5–60.1%.

**RESULTS**

**Analytical value of the quality control samples**

As calculated using the JSCC method, the assigned LDH values of control Wako-I and Wako-II were 152 and 345 U/L, respectively. Meanwhile, in the IFCC method, the assigned LDH values of control Wako-I and Wako-II were 154 and 356 U/L, respectively. In both the JSCC and IFCC methods for LDH analysis, the intra-assay coefficients of variation (CVs) using control Wako-I were 0.45% and 0.74%, respectively, while the inter-assay CVs of control Wako-I were 0.60% and 0.99%, respectively.

**Distribution of LDH measurements in the JSCC and IFCC methods**

The distribution range of LDH measurements in 177 canine plasma samples was 13–202 U/L for the JSCC method and 14–175 U/L for the IFCC method. The median (interquartile range) was 43 U/L (30–68) for the JSCC method and 38 U/L (28–61) for the IFCC method. The number of samples whose LDH measurements fell within the reference range was 160 (90%).

The LDH distribution range in 115 feline samples was 25–300 U/L for the JSCC method and 24–289 U/L for the IFCC method. The median (interquartile range) was 94 U/L (63–140) for the JSCC method and 89 U/L (62–130) for the IFCC method. A total of 101 (88%) samples fell within the reference standard range (JSCC method).

**Ratio of IFCC values to JSCC measurements (IFCC/JSCC ratio)**

The IFCC/JSCC ratio in 177 canine plasma samples ranged from 0.74 to 1.28. The IFCC/JSCC ratio was <1.0 in 151 samples (85%). The IFCC/JSCC ratio did not show statistically significant correlations with age \( (P=0.173) \) or weight \( (P=0.796) \). However, it was significantly correlated with LDH activity by the JSCC \( (r=−0.434, P<0.001) \) and IFCC methods \( (r=−0.341, P<0.001) \). Statistically significant differences in sex \( (P=0.289) \) or breed \( (P=0.545) \) were not observed in the distribution of the IFCC/JSCC ratio.

In felines, the range of IFCC/JSCC ratios was 0.79–1.11, and 101 (88%) samples had IFCC/JSCC ratios <1.0. A statistically significant correlation was not found between the IFCC/JSCC ratio and body weight \( (P=0.625) \). Conversely, significant negative correlations were found between the IFCC/JSCC ratio and age \( (r=−0.200, P=0.032) \), and LDH activity using the JSCC \( (r=−0.438, P<0.001) \) and IFCC methods \( (r=−0.376, P<0.001) \). Group differences were not observed in the IFCC/JSCC ratios for gender \( (P=0.715) \) and feline breed discrimination \( (P=0.413) \).

**Results of plasma LDH isozyme analysis**

The LDH isozyme analysis of 177 canine plasma samples revealed a median (interquartile range) of 14% (11–19) for LDH1, 13% (10–15) for LDH2, 21% (17–24) for LDH3, 16% (14–17) for LDH4, and 33% (27–43) for LDH5. The number of samples within the reference range was 153 for LDH1, 49 for LDH2, 170 for LDH3, 159 for LDH4, and 160 for LDH5.

The median (interquartile range) percentage of each fraction in 115 feline plasma samples were as follows: LDH1, 22% (15–28); LDH2, 11% (8–13); LDH3, 12% (11–15); LDH4, 20% (18–24); and LDH5, 32% (24–40). The number of samples within the feline LDH isozyme reference range was 56 for LDH1, 115 for LDH2, 108 for LDH3, 89 for LDH4, and 102 for LDH5.

**Regression analysis between the JSCC and IFCC method measurements**

In 177 canine samples, the regression equation for the JSCC and IFCC method measurements as \( x \) and \( y \), respectively, was \( y=0.818x + 3.437 \), with a correlation coefficient \( r=0.9909 \) (Fig. 1). The 95% CI of the slope and intercept, which were obtained using the bootstrap method, were 0.787–0.850 and 2.049–5.079, respectively. The regression equation for the samples \( (N=37) \) with LDH activity (JSCC method) and isozyme composition that are all within the reference range was \( y=0.778x + 2.320 \) \( (r=0.9869) \), and the 95% CI for the slope and intercept were 0.737–0.838 and 0.024–4.147, respectively.

In 115 feline samples, the regression equation was \( y=0.933x + 2.051 \) \( (r=0.9983) \) (Fig. 2). The 95% CI of the slope and intercept were 0.915–0.947 and 0.592–3.769, respectively. The regression equation for the samples \( (N=35) \) with LDH activity (JSCC) and isozyme
composition that are all within the reference range was \( y = 0.917x + 3.330 \) (\( r = 0.9961 \)), and the 95% CI for the slope and intercept were 0.887–0.941 and 0.530–6.934, respectively.

**Brand–Altman plot between the IFCC and JSCC measurement values**

The Brand–Altman plots of the JSCC and IFCC measurements in 177 canine and 115 feline samples are shown in Fig. 3 and Fig. 4, respectively. In both species, the difference between the two methods (IFCC – JSCC) on the y-axis increased as the mean value of the two methods on the x-axis increased, indicating the existence of a proportional systematic error between the two measurements. Moreover, the plot was biased towards negative values on the y-axis, indicating that there is a constant systematic error between the two measurements. In canines and felines, each of the 14 and 5 samples, respectively, deviated from the acceptable range of error (mean ± 1.96 SD of difference).
Correlation between the IFCC/JSCC ratio and each LDH isozyme value

The scatterplots of each LDH isozyme % (x) and IFCC/JSCC ratio (y) are shown in Fig. 5 for canines and in Fig. 6 for felines. In both species, the IFCC/JSCC ratio showed a significant positive correlation with LDH1 (canine; r=0.427 and P<0.001, feline; r=0.594 and P<0.001), LDH2 (r=0.628 and P<0.001, r=0.471, P<0.001), and LDH3 (r=0.360 and P<0.001, r=0.188, P<0.05). Conversely, a significant negative correlation was found with LDH5 (r= −0.655, P<0.001) in canines, and LDH4 (r= −0.421, P<0.001) and LDH5 (r= −0.517, P<0.001) in felines.

**DISCUSSION**

The regression equation for the relationship between the LDH measurements of the JSCC (x) and IFCC (y) methods in 177 canine plasma samples was y=0.818x + 3.437. Moreover, 151 samples (85%) had IFCC values below the JSCC value. The slope was even smaller for 37 samples whose LDH activity and isozymes were all within the reference range, y=0.778x + 2.320. Furthermore, the IFCC values were below the JSCC values in 36 (97%) samples. The Brandt–Altman plot indicated the existence of constant and proportional systematic errors between the measurements of the JSCC and IFCC methods. In addition, 14 samples deviated from the acceptable range (mean ± 1.96 SD of difference). The results of this study suggest that the IFCC and JSCC methods may not be equated in canine plasma LDH measurements from an analytical science perspective. However, convenience is also important in the clinical field, and users may decide whether the deviation between the IFCC and JSCC values is acceptable or not.
One way to determine if the deviation between the IFCC and JSCC methods is clinically acceptable is to use the error tolerance limits of the analytical method. In human clinical chemistry, some methods may be used to calculate the acceptable limits of error of analytical methods, such as the Tonks’ error tolerance limits [13]. The following equation is used to determine Tonks’ error tolerance limit for the canine LDH assay:

\[
\text{Tonks error tolerance limit} \% = \frac{\text{upper limit of LD reference range} - \text{lower limit}}{\text{median value of reference range}} \times 100
\]

The tolerance limit is 50%, provided that if the value is >10%, the tolerance limit for the LDH analytical method is 10% because of the 10% tolerance limit principle.

In this study, the number of samples whose IFCC value fell within ± 10% of the JSCC method (i.e., the IFCC/JSCC ratio ranged from 0.90 to 1.10) was 83 (47%). Thus, it may be difficult to clinically equate IFCC and JSCC values for canine LDH because more than half of the values exceeded the Tonks’ error tolerance limits. However, it must be noted that this evaluation method is only one example. Each facility should consider how to treat IFCC and JSCC values and the intended use of the test data.

The regression equation for the relationship between LDH measurements for 115 feline samples was \(y=0.933x + 2.051\). Among these, 101 (88%) samples had IFCC values below the JSCC value. For the 35 specimens whose LDH activity and isozymes were all within the reference range, \(y=0.917x + 3.330\), 30 (86%) had IFCC values below JSCC. The Brandt–Altman plot indicated the existence of constant and proportional systematic errors between the JSCC and IFCC measurements. In addition, five samples deviated from the acceptable range (mean ± 1.96 SD of difference). The results also indicated that the IFCC and JSCC method measurements may not be equated for feline LDH from an analytical science perspective.
For the LDH assay in felines, we also determined the error tolerance limit of Tonks and considered whether the discrepancy between the IFCC and JSCC methods is acceptable for clinical use or not. The error tolerance limit of Tonks for the LDH assay in felines is 10%, which is similar to that in canines. In 112 samples (97%), the IFCC/JSCC ratio fell within the range of 0.90 to 1.10. Most of the specimens in this study fell within the acceptable range of error, suggesting that the IFCC and JSCC can be treated as clinically equivalent values in felines. However, it is not clear whether the samples in the higher value range would fall within the acceptable range.

However, it should be noted that this evaluation method is only an example. In the actual shifting to the IFCC method, it is necessary to consider how to handle IFCC and JSCC values and the intended use of the test data at each facility.

The reason for the discrepancy between the IFCC and JSCC methods may be because both reagents are not equally reactive to each LDH isozyme. Since there is limited knowledge on the reactivity of LDH and assay reagents in the veterinary field, the following discussion is based on the findings for human LDH. The regression equation for the relationship between the JSCC (x) and IFCC methods (y) for human blood LDH is y=0.913x –16.101. Within the reference range, the difference between the JSCC and IFCC methods is small, and the current reference range (124–222 U/L) does not need to be changed [8]. However, the reactivity of both assay reagents to each LDH isozyme is not homogeneous, and a significant change in the isozyme ratio due to a disease that significantly increases some LDH isozymes may cause a discrepancy between the two assay methods [7].

The factors that cause differences in reagent reactivity among LDH isozymes are the subunit composition of LDHs and their optimum pH. The optimum pH of LDH1, which consists only of H-type subunits, is 9.5–10.0, while the optimum pH of LDH5, which consists only of M-type subunits, is 6.0–8.5. Meanwhile, LDH2, 3, and 4 show optimal pH intermediate between LDH1 and LDH5 [12].

The pH of the LDH measurement reagent for the IFCC and JSCC methods are around pH 9.4 (37°C) and pH 8.65 (37°C), respectively. When expressing the reactivity of LDH1 and LDH5 in both assay reagents, LDH1 >LDH5 for the IFCC method reagent and LDH1 <LDH5 for the JSCC method reagent [12]. In other words, LDH activity values are IFCC values >JSCC values for LDH1-dominant specimens and IFCC values <JSCC values for LDH5-dominant specimens. As shown in the results of this study, canines and felines tend to be LDH5-dominant. This tendency is particularly strong in specimens in which LDH activity and isozymes fall within the reference range. Therefore, the IFCC method values generally deviated in the direction of lower values compared to the JSCC method.

We conducted a similar study to the present study with LDH in dairy cattle and determined that LDH1 isozymes are predominant in dairy cattle and that the activity values of LDH are IFCC method >JSCC method [14]. Since the composition of LDH isozymes may differ among animal species, the veterinary field should evaluate the relationship between the IFCC and JSCC method measurements for each animal species.

In canines, JSCC values and LDH1 (r= −0.414, P<0.001), LDH2 (−0.352, P<0.001), and LDH3 (−0.173, P<0.05) showed significant negative correlations, while LDH5 (0.346, P<0.001) showed a significant positive correlation. Furthermore, IFCC values and LDH1 (−0.361, P<0.001) and LDH2 (−0.266, P<0.001) exhibited significant negative correlations, while LDH5 (0.266, P<0.001) showed a significant positive correlation. In felines, JSCC values and LDH1 (r= −0.464, P<0.001) and LDH2 (−0.261, P<0.05) exhibited significant negative correlations, while LDH4 (0.383, P<0.001) and LDH5 (0.294, P<0.01) exhibited significant positive correlations. Significant negative correlations were identified between IFCC values and LDH1 (−0.452, P<0.001) and LDH2 (−0.270, P<0.01), and significant positive correlations were identified for LDH4 (0.370, P<0.001) and LDH5 (0.284, P<0.01). Specifically, isozymes tended to be LDH5-dominant as LDH activity increased in the population in this study. The reason why the IFCC/JSCC ratio decreased with increasing LDH activity in canines and felines may be accounted for by the change in isozyme ratio, although this factor is unknown.

Age negatively correlated with the IFCC/JSCC ratio in felines (−0.200, P<0.05). However, age did not correlate with body weight, IFCC activity, JSCC activity, or LDH1–5 isozyme ratio. Therefore, it may be difficult to explain what causes the correlation between age and IFCC/JSCC in our data.

The limitations of this study include the following. First, specimen bias may have occurred. Since the study utilized blood samples from canines and felines that visited a secondary veterinary clinic for analysis, it can be assumed that none of the population are healthy, and all had some disease. Thus, the results of the analysis cannot be generalized to the general population. Subsequently, regarding the specimen preservation method in this study, plasma was frozen at −80°C for the batch processing of the isozyme analysis. However, fresh plasma is generally used for clinical testing. Although it was confirmed beforehand that plasma LDH activity was not decreased by cryopreservation, it cannot be assured that all specimens were not affected. Furthermore, regarding the measurement reagents, since all commercially available reagents compliant with the IFCC and JSCC methods for human medical use are standardized, the differences among reagent manufacturers do not have to be considered when measuring LDH in humans. However, since there is no standardization for the measurement of LDH in various animals, differences may exist among the reagent manufacturers. It should be noted that since the data in this study were obtained using reagents from one company, different results may be obtained when using reagents from other reagent manufacturers. In any case, when switching to the IFCC method, each facility should check the deviation from the JSCC method and consider whether or not to revise the reference range and clinical judgment value settings.

Overall, the LDH measurements in canines and felines are lower in the IFCC than in the JSCC method. This is presumably because the LDH isozymes in canines and felines are LDH5-dominant. The increase in the systematic errors between the two methods was in the high value range. Moreover, the errors exceeded the upper limit of the reference and allowable ranges in the Brand–Altman plot in canines. The difference also widens at high values in felines; however, the analysis of data beyond the reference range was insufficient for this study. Therefore, when switching to the IFCC method for LDH measurement, it is necessary to consider whether or not to continue using the reference ranges and clinical diagnostic values established based on the conventional JSCC method at each facility. The JSCC method is used only in Japan; thus, caution should be exercised when comparing JSCC values with values published in scientific papers and books from other countries.
CONFLICT OF INTEREST. The authors have no conflicts of interest that are directly relevant to the content of this article.

ACKNOWLEDGMENTS. The authors thank Chie Tanaka and Emi Kan for their help in the biochemical analysis.

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