Determination of serum bone-specific alkaline phosphatase isoenzyme activity in captive Asian elephants (*Elephas maximus*) using an agarose gel electrophoresis method

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ABSTRACT. The bone-specific alkaline phosphatase (ALP) isoenzyme activity was measured in 51 serum samples from four captive Asian elephants (*Elephas maximus*) using a conventional method with wheat germ lectin precipitation and a commercial agarose gel electrophoresis (AGE) kit; the isoenzymes were designated as bone-specific ALP (BAP) and ALP isoenzyme 3 (ALP3), respectively. This study examined the suitability of the AGE kit for analyzing blood biochemistry in Asian elephants. The serum ALP3 and BAP activities were strongly positively correlated and met the evaluation criteria for agreement using Bland-Altman analysis. The results indicate that the AGE kit can be used to examine the blood biochemistry in Asian elephants instead of the conventional method.

KEY WORDS: agarose gel electrophoresis (AGE), Asian elephants (*Elephas maximus*), bone markers, bone-specific alkaline phosphatase isoenzyme

Musculoskeletal disorders often occur in captive elephants [10, 14]. Given the massive weight of elephants, these diseases often require prolonged treatment. Metabolic bone diseases, which may result from dietary mismanagement, poor nutrition, or intestinal malabsorption, can cause skeletal disorders in young, growing elephants [7]. Bone metabolism markers in blood are useful for the early diagnosis of metabolic bone diseases and several studies have reported the baseline levels and age-related fluctuation of several such markers in the blood of Asian elephants (*Elephas maximus*) [2, 15, 22]. Osteocalcin (OC) and bone-specific alkaline phosphatase (BAP) were measured in Asian elephants as bone formation marker and indicated its usability [2, 15]. However, OC is known to be affected by factors such as renal disorder and vitamin K deficiency [18].

BAP is an isoenzyme of alkaline phosphatase (ALP). The activity of this isoenzyme predicts bone formation status due to osteoblastic activity without affected by hepatic or renal failure [16]. BAP measurement is used for veterinary medicine, such as the effect of exercise, fracture healing and skeletal neoplasia in canine, equine, and bovine [1, 8]. In Asian elephant, BAP is used for age-related trends in bone formation and evaluating bone metabolism in collaboration with bone resorption marker measurement, and it indicated to contribute to the health management [2, 22]. Studies conducted in Thailand [2] and Japan [22] measured the serum BAP activity in Asian elephants with a spectrophotometric method using the wheat germ lectin precipitation (WGLP) technique [3]. However, the WGLP technique requires technical expertise and expensive materials [17] and is likely subject to variation across batches of wheat germ [3].

To the best of our knowledge, ALP isoenzymes determination in elephants have not been reported. It was only said ALP occurs in all tissues with the highest levels in liver, bone, kidney, intestine and placenta in elephants like other mammals [7].Six ALP isoenzymes are found in human blood and tissues: high-molecular-weight ALP (ALP1), hepatic ALP (ALP2), bone ALP (ALP3), placental ALP (ALP4), intestinal ALP (ALP5), and IgG-bound ALP (ALP6) [12]. The isoenzymes have different physicochemical

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and electrophoretic properties. Of the six, ALP2 and ALP3 are the most prominent in blood [9, 13, 21]. In Japan, many private clinical laboratories use commercial agarose gel electrophoresis (AGE) kits to evaluate serum ALP isoenzymes in human serum samples. The use of this off-the-shelf kit may also benefit veterinary practice.

In this study, the bone-specific isoenzyme activity of ALP in serum samples from Asian elephants was measured using the WGLP technique and an AGE kit, to determine the feasibility of using the AGE kit for measuring this biomarker in this species. The respective isoenzymes determined with the WGLP and AGE methods were designated BAP and ALP3.

This study examined 51 serum samples obtained from four clinically healthy Asian elephants (A–D) at the Ichihara Elephant Kingdom. Blood samples were taken monthly for medical check-ups from elephants A (non-pregnant female; n=16, at ages 9.5–10.8 years), B (non-pregnant female; n=14, at ages 3.5–4.6 years), C (male; n=10, at ages 2.7–4.2 years), and D (non-pregnant female; n=11, at ages 31.0–32.3 years). Blood was withdrawn from an auricular vein using a 5-ml disposable syringe (Nipro Medical, Osaka, Japan) with a 23 G butterfly needle (Top Medical, Tokyo, Japan) and transferred to a blood-collection tube (GP-SP1029, Nipro Neo-tube; Nipro Medical). After the blood clotted, the samples were centrifuged and the serum was frozen at −20°C until analysis.

The serum BAP activity was measured spectrophotometrically using the WGLP technique [3]. Briefly, 30 μl of serum sample was pre-incubated with 3 μl of diluted Triton-X (Wako Pure Chemical Industries), then 30 μl of wheat germ lectin (lectin from Triticum vulgaris; Sigma-Aldrich, St. Louis, MO, U.S.A.) solution (5 mg/ml) was added and incubated at 37°C for 30 min. The mixtures were cooled on ice and centrifuged at 2,000 ×g for 10 min; then, duplicate 20 μl aliquots were removed to measure lectin-insensitive ALP isoenzyme levels. Serum activities of total ALP and the lectin-insensitive ALP isoenzyme were determined using a LabAssay ALP kit (Wako Pure Chemical Industries). BAP was calculated by subtracting the activity of the lectin-insensitive aliquot from the total activity of the untreated sample.

To measure the serum ALP3 activity, AGE was performed using a QuickGel ALP agarose gel kit (J713; Helena Laboratories Japan, Saitama, Japan), QuickGel ALP (bone type) reagent (J871; Helena Laboratories Japan), and a fully automatic electrophoresis analyzer (Epalyzer-2; Helena Laboratories Japan), according to the manufacturer's instructions. To verify the fractions of ALP isoenzyme, human control serum (Lot. 118J-5104; Helena Laboratories Japan) was used as a reference for the two major fractions, ALP2 and ALP3. Each 30-μl serum sample was incubated with 4 μl of a 1.5 U/ml protease solution and 2 μl of separator solution containing neuraminidase at room temperature for 30 min. The gels were electrophoresed at 230 V for 23 min at 15°C, stained with nitro blue tetrazolium, and then scanned. The ALP isoenzyme fractions were assessed using Edbank III software (Helena Laboratories). Each electrophoretogram was examined visually, and the ALP isoenzyme fractions were identified. The relative ALP3 activity was determined as the optical absorbance percentage, and the absolute activity (U/l) was calculated using the total ALP activity.

Numerical data are expressed as the mean ± standard deviation. The serum ALP3 and BAP activities were compared following standard recommendations for comparing analytical techniques based on Deming's regression and Bland–Altman analysis. In the Bland–Altman analysis, the limit of agreement (LoA) was adjusted for repeat sampling using a reported calculation [4] because 10–16 samples were collected from each animal. In addition, the consistency of the two parameters was judged according to the criteria of Critchley and Critchley [5] and calculated as the percentage LoA of less than 20%. The level of significance was set at $P<0.05$. All statistical analyses were performed using Prism ver. 6 for Windows (GraphPad Software, La Jolla, CA, U.S.A.).

Figure 1 shows an agarose gel image (a) and representative electrophoretograms of human control serum (b) and four serum samples from elephants A–D (c–f). During electrophoresis (Fig. 1a), each elephant serum sample (lanes 2–5) revealed separate fractions corresponding to ALP2 and ALP3, as in the human control serum (lane 1). From the electrophoretograms, the samples from elephants A–D were found to contain 24.8 ± 4.1, 18.4 ± 5.8, 17.0 ± 2.0, and 46.6 ± 3.5% ALP2 and 75.2 ± 4.1, 81.6 ± 5.8, 82.9 ± 2.0, and 53.4 ± 3.5% ALP3, respectively.

Figure 2 shows a scatterplot (a) of the serum ALP3 and BAP activities and the Bland–Altman plot (b) showing the differences in the activities of ALP3 and BAP in the 51 serum samples from the four Asian elephants. Deming's regression slopes between the serum ALP3 and BAP activities were 1.0 for all estimated values and the intercepts were acceptable at 5.3 (Fig. 2a). The serum ALP3 and BAP activities were well correlated (r=0.98, $P<0.0001$). All points obtained based on the Bland and Altman [4] bias presentation were within the agreement plots (Fig. 2b). The percentage LoA between the ALP3 and BAP activities [5] was 1.0% [(3.38/344.73) ×100].

In comparison of the individual difference, serum ALP3 activities in elephant D (147.5 ± 25.5 U/l, n=11) were significantly lower ($P<0.001$) than those in other three elephants [329.9 ± 71.4 U/l in elephant A (n=16), 475.0 ± 120.6 U/l in elephant B (n=14) and 450.7 ± 76.6 U/l in elephant C (n=10)]. The ALP3 activities in elephant B and C were significantly higher ($P<0.001$) than those in elephants A.

The bone-specific isoenzyme ALP is a non-collagenous plasma membrane glycoprotein secreted by osteoblasts that is essential for bone mineralization and is a highly specific marker of osteoblast function [6]. In Asian elephants, the serum BAP activities were traditionally measured using the WGLP technique [2, 22]. In this study, the Deming regression analysis revealed a significant positive correlation between the serum ALP3 and BAP activities and a well-fitted slope (y=1.0x + 5.3, $P<0.0001$). In the Bland–Altman analysis, the range of the 95% LoA between ALP3 and BAP accounted for 1.0% of the average of BAP. These results satisfied the evaluation criteria for agreement between two medical measurement methods using Bland–Altman analysis [5]. Hence, the serum ALP3 and BAP activities in each serum sample from the four Asian elephants were essentially equal and highly interchangeable. Therefore, serum ALP3 activity is a useful indicator of bone formation in Asian elephants, and can be used as a substitute for serum BAP activity.
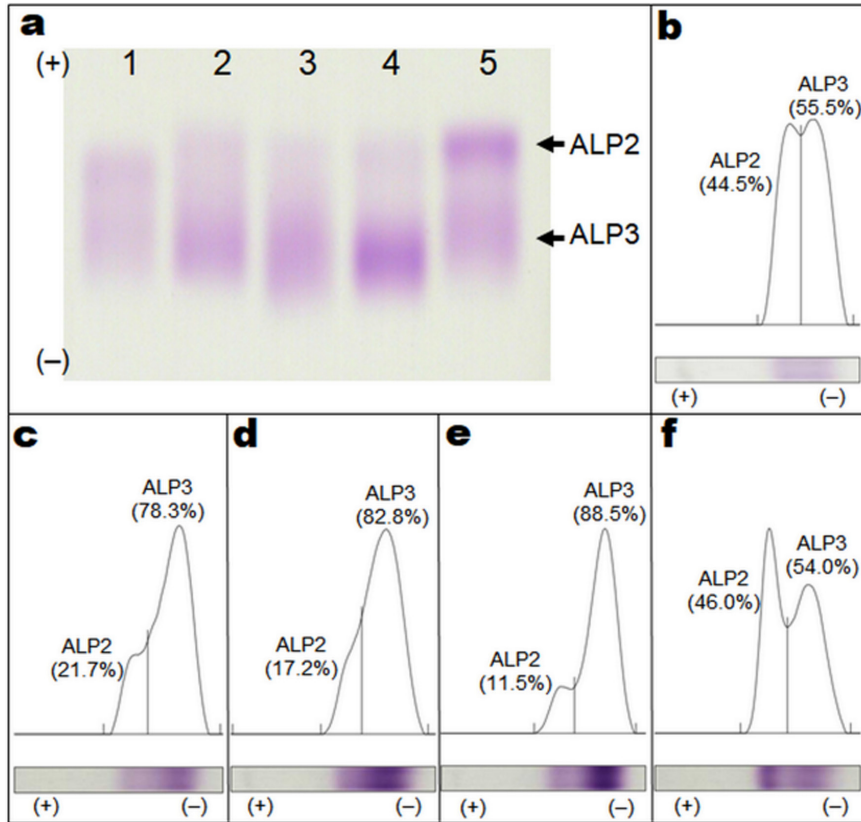
Fig. 1. Photograph of agarose gel electrophoresis (a) and representative densitometer trace pattern (electrophoretogram) images (b–f) of the serum samples from four elephants. (a) Lane 1, human control serum (Helena, Lot. 118J-5104); lane 2, elephant A at the age of 10.8 years; lane 3, elephant B at the age of 4.6 years; lane 4, elephant C at the age of 3.8 years; and lane 5, elephant D at the age of 32.2 years. Electrophoretograms of lanes 1 (b), 2 (c), 3 (d), 4 (e), and 5 (f).
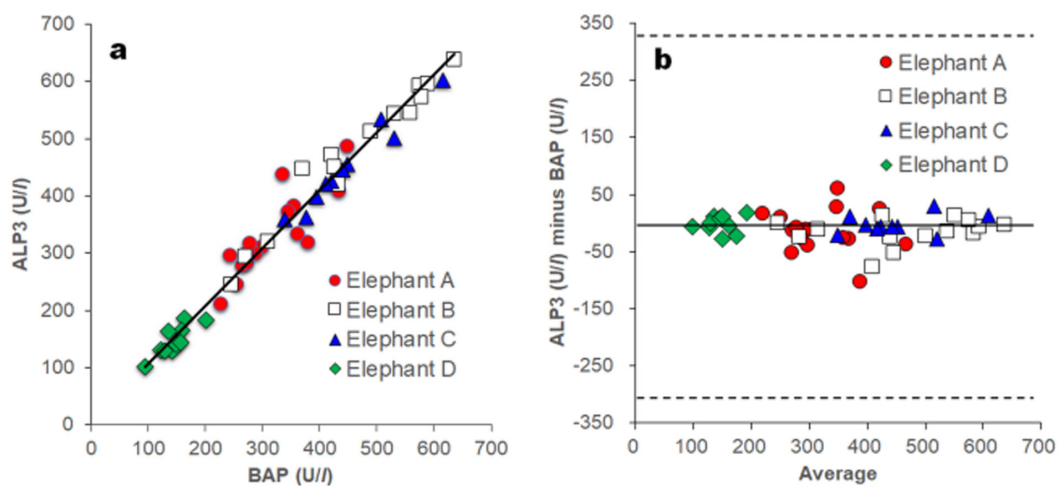

Fig. 2. Scatterplot (a) and Bland-Altman plot (b) of the serum ALP3 and BAP activities in samples from four Asian elephants (n=51). (a) Deming's regression: $y=1.0x + 5.3$ ($r=0.98$, $P<0.0001$). (b) Mean bias (solid line): -9.3. The 95% confidence limits of agreement are -330.9–312.2 (dotted lines).

In electrophoretic analyses of human serum samples, the clinical value of ALP isoenzymes is limited by the inability to separate ALP2 and ALP3 [21]. A band resulting from high-molecular mass intestinal ALP (HIALP) often overlaps the ALP3 band on AGE [19]. To resolve this problem, the AGE kit used in this study requires pretreatment of the serum with protease and neuraminidase before electrophoresis. The protease pretreatment induces the conversion of HIALP into ALP5, which enables the clear

discrimination of ALP3 on the agarose gel [19]. Neuraminidase pretreatment makes it possible to distinguish the ALP2 and 3 bands based on the different mobilities of each isoenzyme, allowing densitometric quantification [21]. In the present study, the AGE kit distinguished the ALP 2 and 3 bands for all of the Asian elephant serum samples, matching the electrophoretic pattern of the human control serum (Fig. 1). Therefore, we believe that the AGE kit used in this study can be used to evaluate the ALP isoenzyme patterns and quantify each isoenzyme in the serum samples of Asian elephants.

Previous studies have reported that serum BAP activities have a negative correlation with ages in Asian elephants [2, 22]. The present study revealed the similar finding that serum ALP3 activities in young elephants were higher than those in old animals. Generally, it has been reported that the blood levels of BAP and other bone markers may be influenced by factors, such as age, gender, nutrition and the time of blood sampling [11]. We previously reported that serum activities of BAP and tartrate-resistant acid phosphatase 5b (an osteoclastic bone resorption marker) [20] and those ratio in a bull and a male calf elephant were different from other females [22]. It is also known that serum ALP activity fluctuates in mature male elephants during the musth period (a phase of elevated androgen levels and heightened sexual activity) [7]. Therefore, further studies are necessary to evaluate the circulatory levels of ALP3 and bone metabolic status in elephants with various physiological conditions.

In conclusion, the serum ALP3 activity can substitute for the serum BAP activity and act as an indicator of bone-specific ALP isoenzymes in Asian elephants.

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