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*Short communication*

# Determination of plasma bone-specific alkaline phosphatase isoenzyme activity in Holstein calves using a commercial agarose gel electrophoresis kit

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## Abstract

We measured the bone-specific alkaline phosphatase (ALP) isoenzyme activity in 67 plasma samples from 14 newborn Holstein calves using both a conventional method (featuring heat inactivation) and a commercial agarose gel electrophoresis (AGE) kit; the relevant isoenzymes were termed bone-specific ALP (BAP) and ALP isoenzyme 3 (ALP3). We explored whether the AGE kit afforded reliable data when used to analyze samples from Holstein calves. The blood was collected from the jugular vein of each calf immediately prior to the first colostrum feeding (pre-feeding), 20 and 40 h after pre-feeding, and on days 4 and 7; whereas three samples (from three calves) were not obtained. The total plasma ALP activity varied widely, exceeding the ranges of reference values. On electrophoresis, 52 of 67 plasma samples (77.6 %) clearly contained both ALP isoenzyme 2 and ALP3, as did control human serum. The total ALP activity of the 52 samples ranged from 166–1989 U/L (median: 1013 U/L), whereas the values for the other 15 samples (22.4%) exhibiting abnormal isoenzyme fractionation ranged from 1014–5118 U/L (median: 1780 U/L). In the 52 plasma samples exhibiting clearly separated isoenzymes, ALP3 and BAP activities were strongly positively correlated as revealed by Deming regression ( $y = 0.93x + 22.6$ ,  $p < 0.0001$ ) and Bland-Altman analysis (ALP3/BAP activities limit of agreement:  $-5.1\%$ ). Thus, the AGE kit yields useful information on newborn calves, and can replace the conventional method when the total plasma ALP activity is less than approximately 1000 U/L.

**Key words:** agarose gel electrophoresis (AGE), bone-specific alkaline phosphatase isoenzyme, calf

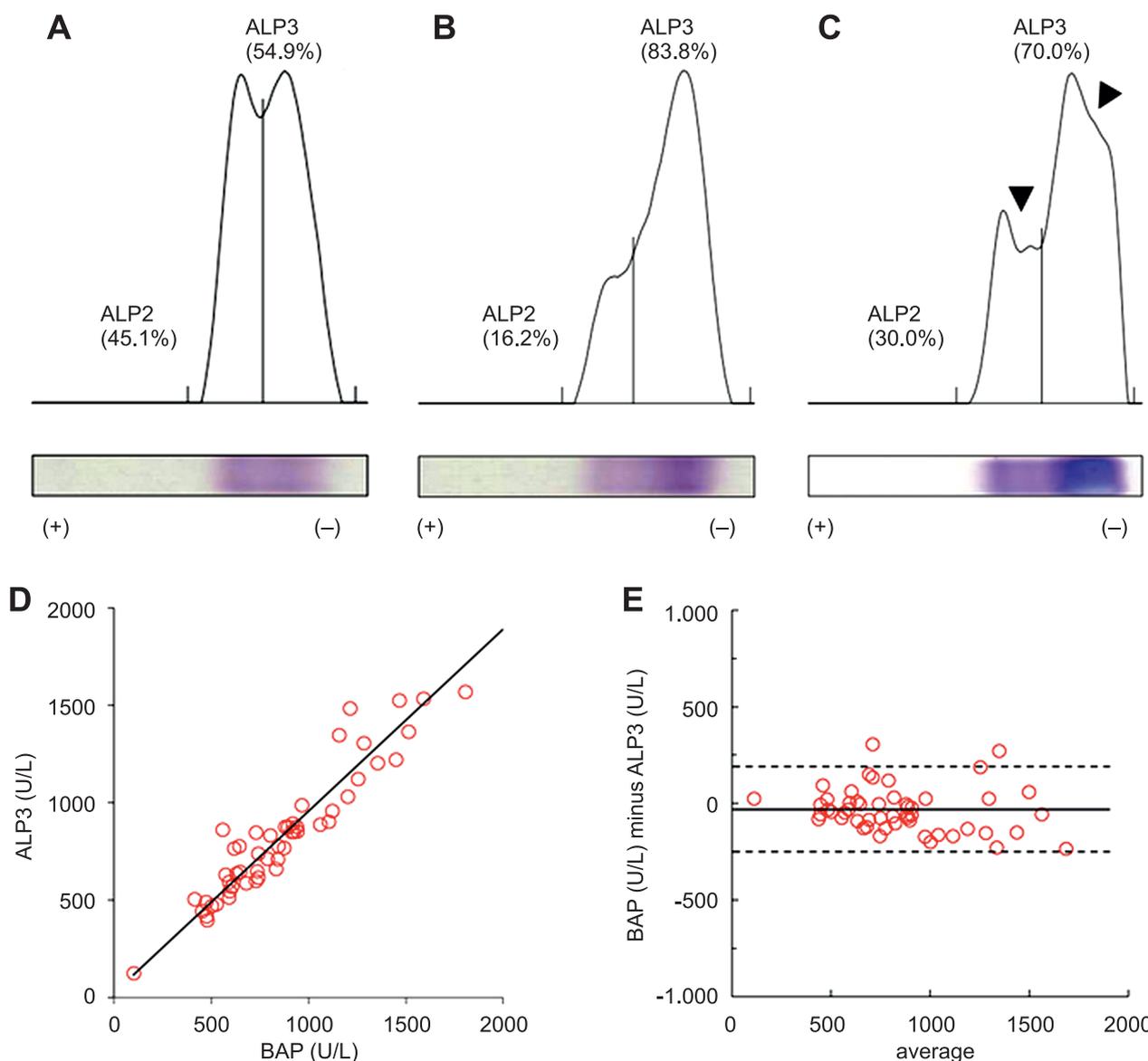


Fig. 1. Representative densitometric (electrophoretic) traces of plasma samples (A–C) and scatter (D) and Bland-Altman (E) plots of plasma ALP3 and BAP activities in 14 newborn calves. (A) Human control serum (Helena, lot no. 118J-5104). (B) A plasma sample from one calf (total ALP activity: 605 U/L). (C) A plasma sample from another calf 20 h after the first colostrum feeding (total ALP activity: 4813 U/L). Note the distortions (arrowheads). ALP2, ALP isoenzyme 2; ALP3, ALP isoenzyme 3. (D) The Deming regression slopes of 52 exhibiting clearly separated isoenzymes are  $y = 0.93x + 22.6$  ( $p < 0.0001$ ). (E) The mean biases (solid lines) with 95% confidence limits of agreement (dotted lines) in 52 exhibiting clearly separated isoenzymes are  $-33.4$  with  $-259.6$  to  $192.9$ .

## Introduction

Bone-specific alkaline phosphatase (BAP) is an isoenzyme of alkaline phosphatase (ALP). In cattle, circulating BAP levels are used to monitor trends in bone formation with age (Yamagishi et al. 2009), and, combined with other markers, bone metabolism/resorption in the neonatal period (Hatate et al. 2019) and during parturition (Devkota et al. 2013). BAP levels in cattle have traditionally been measured spectrophotometrically employing heat-inactivation (HI) or the wheat germ lectin precipitation (WGLP) technique. The HI technique is preferable (Mohebbi et al. 2010)

as it is almost twice as sensitive as the WGLP technique and preparation of the calibration standards is simple (Farley et al. 1993).

A recent commercial agarose gel electrophoresis (AGE) kit can be used to analyze human ALP isoenzymes. This off-the-shelf kit may be useful in veterinary practice. Here, we used the HI technique and the commercial AGE kit to measure bone-specific ALP isoenzyme activities in plasma samples from Holstein calves; we explored whether the kit could be used to measure the levels of this biomarker in cattle. The isoenzymes assayed using the HI and AGE methods were designated BAP and ALP3, respectively.

## Materials and Methods

We assayed 67 frozen ( $-60^{\circ}\text{C}$ ) plasma samples collected from 14 neonatal calves in our previous study (Hatate et al. 2019). In that study, blood was collected from the jugular veins immediately prior to the first colostrum feeding (pre-feeding), 20 and 40 h after pre-feeding, and on days 4 and 7. Here, we sought to analyze all samples; however, because we lacked three samples (from three calves), we thus assayed 67 samples. Total plasma ALP and BAP activities were spectrophotometrically measured using a LabAssay ALP kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). To measure plasma BAP activity using the HI method, plasma samples were incubated at  $56^{\circ}\text{C}$  for 15 min (Mohebbi et al. 2010). Plasma ALP3 activity was measured via AGE performed using a QuickGel ALP agarose gel kit (catalog no. J713; Helena Laboratories Japan, Saitama, Japan), the QuickGel ALP (bone-type) reagent (catalog no. J871; Helena Laboratories), and an automatic electrophoresis system (Epalyzer-2; Helena Laboratories). A human control serum (Lot 118J-5104; Helena Laboratories) containing ALP isoenzyme 2 (ALP2) and ALP3 served as a reference for the two major isoenzymes. Sample ( $30\ \mu\text{L}$ ) was incubated with  $4\ \mu\text{L}$  of a  $300\ \text{U/mL}$  protease cocktail and  $2\ \mu\text{L}$  of separator solution containing neuraminidase, at room temperature for 30 min. Electrophoresis was performed for 23 min at 230 V and  $15^{\circ}\text{C}$ ; protein bands were stained with nitroblue tetrazolium and the gels scanned. ALP isoenzyme levels were quantitated with the aid of Edbank III software (Helena Laboratories). All ALP isoenzyme bands were visually identified. The relative ALP3 activity was calculated as a percentage of the optical absorbance of that band; the absolute activity (U/L) was the total ALP activity.

Plasma ALP3 and BAP activities were compared using Deming regression technique and Bland-Altman analysis. Each limit of agreement (LoA) was adjusted to reflect the fact that repeat sampling was in play (Bland and Altman, 2007); we assayed four or five samples from each animal. The two values derived were deemed to be consistent if the percentage LoA was  $<20\%$  using the criteria of Critchley and Critchley (1999). The level of significance was set at  $p<0.05$ . All statistical analyses were performed using Prism ver. 6 software for Windows (GraphPad Software, La Jolla, CA, USA).

## Results and Discussion

Figure 1 shows representative electrophoretograms of human control serum (A) and plasma samples from newborn calves (B and C). During electrophoresis,

52 of 67 plasma samples (77.6%) yielded two distinct bands corresponding to ALP2 and ALP3 (Fig. 1B), as did the human control serum (Fig. 1A). The total ALP activities of these 52 samples were 166–1989 U/L (median: 1013 U/L). The other 15 samples (22.4%) exhibited distorted densitometer trace patterns (Fig. 1C). The total ALP activities of these samples were 1014–5118 U/L (median: 1780 U/L), thus higher than those of plasma samples affording clearly separated isoenzymes. For statistical analysis, 52 plasma samples that yielded clearly separated isoenzyme patterns were analyzed. The Deming regression slope was  $y = 0.93x + 22.6$  ( $p<0.0001$ , Fig. 1D), and 96.2% (50/52) of the points obtained after Bland-Altman bias evaluation were within the boundaries of agreement (Fig. 1E). The percentage LoA between the ALP3 and BAP levels was  $-5.1\%$  [ $(-41.7/825.5) \times 100$ ]. These results satisfied the Bland-Altman criteria for agreement between the two measurement methods (Critchley and Critchley 1999).

The reference level of total circulating ALP in healthy animals is 100–200 U/L for adult dairy cows (Cozzi et al. 2011) and 100–700 U/L for beef cattle aged 1 day to 1 year (Doornenbal et al. 1988). In newborn calves, the total ALP activity increases transiently after colostrum intake, suggesting absorption of colostrum ALP (Hatate et al. 2019). In growing animals, a large proportion of the ALP originates from bone; therefore, higher circulating levels of total ALP are indicative of rapid skeletal growth (Doornenbal et al. 1988). In the present samples, the total plasma ALP activity varied widely within but also outside the reference ranges (166–5118 U/L). A total ALP activity  $>1000\ \text{U/L}$  was associated with distortion of isoenzyme patterns on electrophoretograms, caused by excessively dense staining. Thus, the AGE kit can be used to measure ALP3 levels in newborn calves.

In conclusion, the plasma ALP3 and BAP activities in newborn calves were similar when the total ALP activity was less than approximately 1000 U/L. The plasma ALP3 level may be a useful indicator of bone formation in cattle, serving as a surrogate for BAP activity. The AGE kit conveniently measures the levels of circulating ALP isoenzymes in bovine practice; private clinical laboratories may accept both bovine and human samples.

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