



Validation of the bovine blood calcium checker as a rapid and simple measuring tool for the ionized calcium concentration in cattle

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ABSTRACT. Point-of-care (POC) devices that veterinary practitioners can use to easily and rapidly measure blood ionized calcium (iCa) levels in cows immediately after withdrawing a blood sample on the dairy farm are needed. Aims of present studies was to compare the commercially available ion-selective electrode handheld iCa meter (bovine blood iCa checker) with the benchtop blood gas analyzer GEM premier 3500 and handheld analyzer i-STAT 1. Sixty-two paired-point whole blood samples were obtained from three cows with hypocalcemia experimentally induced by Na₂-EDTA infusion. Whole blood samples were also obtained from the 36 cows kept on a farm in field conditions. The results using the bovine blood iCa checker correlated with those using the GEM premier 3500 and i-STAT 1. Bovine blood iCa checker was “compatible” with the GEM premier 3500 and i-STAT 1 because the frequency of differences between the measurements within $\pm 20\%$ of the mean were 100% (65/65, >75%) and 90.8% (59/65, >75%), respectively. In the field trial, the blood iCa concentration measured by the bovine blood Ca checker was significantly positively correlated with that measured by the i-STAT 1 portable analyzer. Bovine blood iCa checker was “compatible” with the i-STAT 1 because the frequency of differences between the measurements within $\pm 20\%$ of the mean was 100% (36/36, >75%). Results from these findings, the bovine blood iCa checker may be applied as a simplified system to measure the iCa concentration in bovine whole blood.

KEY WORDS: cattle, hypocalcemia, ion-selective electrode, ionized calcium, point-of-care

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Hypocalcemia is a metabolic disorder in which homeostatic mechanisms fail to maintain normal blood Ca concentrations at the onset of lactation [20, 29]. Although the incidence of clinical milk fever in the United States is approximately 5%, as many as 50% of periparturient dairy cows may have subclinical hypocalcemia, with total blood Ca concentrations being between 1.38 and 2.0 mM [7, 18]. As intravenous 22.5% calcium borogluconate infusion immediately induces a state of hypercalcemia, followed by reduction of the whole blood ionized calcium (iCa) and serum total calcium (tCa) concentrations 24 hr later [28]. Therefore, it is difficult to maintain an ideal blood calcium level without monitoring the blood calcium level [11].

Blood calcium exists in 3 fractions as follows: iCa, complexed (cCa), and protein-bound calcium (pCa) [23]. Indeed, Martinez *et al.* [21] defined subclinical hypocalcemia as a blood iCa <1.0 mM without recumbence. Although iCa is biologically active, clinicians rely on serum tCa measurements to predict iCa status. Variations in pCa or cCa result in abnormal tCa concentrations even though the iCa fraction is within normal limits [25]. Agnes *et al.* [1] demonstrated that the correlation coefficients were too low to be usable for prediction of the levels of ionized calcium from the levels of total calcium despite the calves aged 1–6 months were significant positive correlations between ionized and total calcium. Indeed, variation in the relationship between iCa and reference tCa in the days following parturition suggest that these measures cannot be used interchangeably as indicators of Ca status [18]. Therefore, if the iCa concentration in blood can be measured quickly and easily beside the cow, it will greatly improve

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the clinical diagnosis and treatment of hypocalcemia.

The blood iCa concentration is generally measured using conventional blood gas analyzers in diagnostic laboratories and teaching animal hospitals, which is not suitable for farm animal practice. Point-of-care (POC) devices that veterinary practitioners can use to easily and rapidly measure blood iCa levels in cows immediately after withdrawing a blood sample on the dairy farm are needed. The i-STAT 1 (Abbott Point-of-Care, Abbott Laboratories, Chicago, IL, USA) is a popular POC analyzer for bovine practice [4] that measures blood gases, glucose [24], L-lactate [12, 17] and several electrolytes including K^+ , Na^+ , Cl^- and iCa concentrations with gas- and ion-selective electrode potentiometry. A recent validation study revealed that the i-STAT 1 was suitable for measuring the iCa concentration in whole blood [31]. On the other hand, an ion-selective electrode handheld meter, the bovine blood iCa checker (LAQUAtwin-Ca-11C, HORIBA Advanced Techno, Kyoto, Japan), recently became available for measurement of the iCa concentration in whole blood. These handheld meters have the potential to facilitate cow-side diagnosis and treatment of clinical and subclinical hypocalcemia [23]. However, accuracy control is required to use POC devices with the ion-selective electrode method to measure iCa to diagnose hypocalcemia in cows. The objective of this study was to assess an optimized ion-selective electrode Ca-module prototype, bovine blood iCa checker, as a potential cow-side device for iCa measurements in bovine blood within the broad iCa range expected for hypocalcemic cows. In this study, the accuracy of the bovine blood iCa checker and handheld gas analyzer i-STAT 1 were evaluated using the laboratory benchtop blood gas analyzer GEM premier 3500 (Werfen Group IVD, Barcelona, Spain) as a gold standard method. After completing the laboratory-level precision and accuracy control tests of bovine blood iCa checker and handheld gas analyzer i-STAT 1, we evaluated whether bovine blood iCa checker is useful as a POC device in bovine clinical practice using i-STAT 1 as control.

MATERIALS AND METHODS

All procedures were performed in accordance with the Good for the Care and Use of Laboratory Animals of the School of Veterinary Medicine at Rakuno Gakuen University (Approved #: VH15C18). This series of studies consisted of two parts: (1) laboratory-level precision and accuracy control tests, and (2) clinical trials carried out in the northeast and southwest regions in Japan.

In this study, the GEM premier 3500 and i-STAT 1 were used to evaluate the accuracy and clinical usefulness of the bovine blood iCa checker for measuring iCa levels in cattle. The measurement range and the recommended environment for the three iCa concentration measuring instruments used in this study are summarized in Table 1. These values were taken from the manufacturer's instruction manual.

Laboratory-level precision and accuracy control tests

Three non-pregnant, non-lactating, and multiparous cows (731 ± 27 kg, 7.0 ± 0.9 -year-old; mean \pm SD) were enrolled in this study. During the experiment, the cows were housed in a stanchion barn at the university. Three mature cows received aqueous solution containing 5% disodium ethylenediaminetetraacetate (Na_2 -EDTA, Wako Pure Chemical Industries, Osaka, Japan) intravenously (IV) at a flow rate of 1.2 ml/kg/hr. Blood samples were taken before and at 10 min intervals up to 3 hr after the initiation of Na_2 -EDTA infusion. Na_2 -EDTA has been used in infusion studies in cows and other species for more than 35 years, primarily for the induction of hypocalcemia as a model for milk fever [14, 19, 22, 26]. Na_2 -EDTA infusion may therefore be a valid model of spontaneous hypocalcemia [26]. In the hypocalcemic cow model, an infusion rate of 1.2 ml/kg/hr of a 5% (w/v) solution has been suggested as the standard [14, 15, 19, 22].

A total 65 point-paired were collected from cows that underwent Na_2 -EDTA infusion at any time (21, 22 and 22 point-paired blood samples from 3 cows, respectively). The iCa determinations was performed in anaerobically handled whole blood samples with paying attention to dead space. Whole Blood samples were collected via jugular vein venipuncture using 18-gauge regular bevel needle and 5 ml syringe (Terumo Co., Ltd., Tokyo, Japan). The syringe was added heparin sodium solution (AY Pharma, Co., Ltd., Tokyo, Japan) that the final concentration of 10 IU/ml when 5 ml of blood was filled (50 μ l of 1,000 IU/ml heparin sodium solution / syringe). Immediately after blood collection and rolling syringes between the hand to mixing blood and anticoagulant (≤ 5 min), sample were measured iCa concentration using each test device at same time. The concentration range of iCa measured using the desktop blood gas analyzer was 0.24 to 1.22 mM. This range is appropriate because it includes low iCa concentrations from cows with severe hypocalcemia cattle to normal concentrations in healthy cows.

The appearance and operation of the bovine blood iCa checker are shown in Fig. 1. Immediately before measuring iCa by these analyzers, a drop of blood (more than 0.3 ml) was applied directly to the end of a test strip for the bovine blood iCa checker from

Table 1. Performance comparison of the ionized calcium concentration measuring device used in this study

| Type | | GEM premier 3500 | i-STAT 1 | Bovine blood iCa checker |
|-------------------|-----------------------------|------------------|--------------|--------------------------|
| | | The desktop | The handheld | |
| Ionized calcium | Range (mM) | 0.10–5.00 | 0.25–2.50 | 0.1–5.0 |
| Usage environment | Temperature ($^{\circ}C$) | 15–35 | 17–30 | 5–40 |
| | Humidity (%) | 5–90 | <90 | <85 |

These values were taken from data described the instruction manual by each manufacturer.

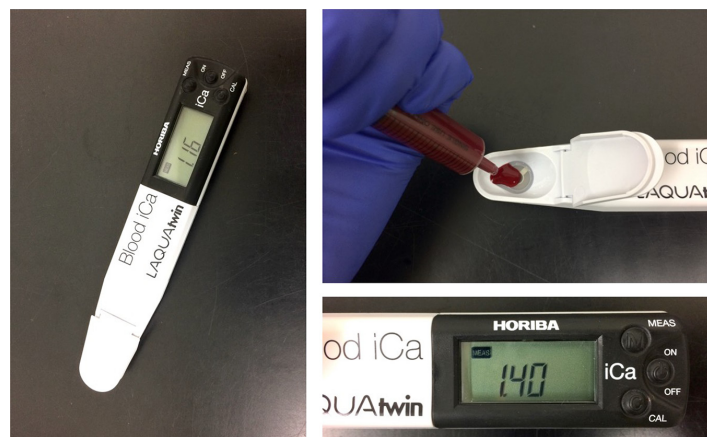


Fig. 1. The bovine blood iCa checker (HORIBA LAQUAtwin-Ca-11C). The appearance of the bovine blood iCa checker (left). Drop 0.3 or more milliliters of blood onto the sensor (right upper), close the light cover, and press the MEAS button to measure the iCa concentration and display the results (right bottom). It takes less than 3 min from initiation of calibration to display the results.

a syringe. The MEAS button was pressed immediately after closing the light cover of the bovine blood iCa checker, and the results appeared on the display immediately. This POC instrument has a measurement range of 0.1 to 5.0 mM and is calibrated by 2-point calibration (1.25 and 2.50 mM) before measurement. It takes less than 3 min from initiation of the calibration to display the results. Blood iCa measurement by the bovine blood iCa checker was performed in duplicate, and the mean value was used for statistics. The median value of the coefficient of variation for iCa measurement was 0.04 mM and the range were 0.00 to 0.12 mM.

The clinical trials carried out used cattle with or without hypocalcemia in the bovine farm

Clinical trials were conducted in two locations, the northern (Kushiro City, Hokkaido, latitude of 42°59' 5.47"N and a longitude of 144°22'52.88"E) and the southern regions of Japan (Ishigaki City, Okinawa Prefecture, latitude of 24°28'0.12"N and a longitude of 124°13'58.80"E). The duration (average temperature [range]) of clinical trials in the northern and southern regions were March-May (7.1°C, [-2°C to 17°C]) and July-August 2019 (30.1°C, [27°C to 34°C]), respectively. In the clinical trials, a total of 36 blood samples drawn from 6 and 3 commercial farms in the northern and southern regions, respectively, were used to measure the iCa concentration by the bovine blood iCa checker and i-STAT 1 portable analyzer. In a clinical trial conducted in the northern region of Japan, total 16 Holstein dairy cows were enrolled and consisted 6 cows immediately after calving, 6 cows being treated for hypocalcemia within 1 week after calving, and 4 healthy cows. In a clinical trial conducted in the southern region of Japan, total 20 Japanese black beef cows were enrolled and consisted 4 cows before expected calving within 1 week, 5 cows after calving within 5 days, and 12 cows that were patients but not hypocalcemia.

The ranges of the outside air temperature at the time of blood collection and blood iCa concentration using the bovine blood iCa checker were -2 to 34°C and 0.370 to 1.260 mM, respectively. The i-STAT 1 was used under the temperature environment of 17 to 30°C, whereas the bovine blood iCa checker was used beside cows regardless of the outside temperature. Therefore, the measurement of blood iCa concentration by the i-STAT 1 portable analyzer was performed beside the cow if the outside air temperature was within 17 to 30°C. However, blood iCa measurement by i-STAT 1 was conducted in an office room of farm with a controlled temperature environment of 17 to 30°C if outside temperature is otherwise.

Statistical analysis

Data were analyzed statistically by Deming regression and Bland-Altman plot using a commercial statistical software program (XLSTAT ver.2020.3 software program, New York, NY, USA). The Bland-Altman plot as the limits of agreement method for assessing the agreement between two methods of medical measurement is widely used. In the clinical trial, pairs of observations using the two methods are independent, Bland-Altman plot analysis were used to assessing the residuals. However, laboratory-level precision trials obtained repeated pairs of measurements on the same subject. Therefore, repeated Bland-Altman plot were performed using an above commercial statistical software to analyzing such clustered observations, both when the underlying quantity is assumed to be changing and when it is not [3, 13, 32]. A repeated Bland-Altman approach is to average the repeated measurements for each subject and use only number of subject's pairs of means to calculate the 95% limits [3]. These limits are narrower than the correct ones. There is little difference because there is much more variation between the subjects than for the repeated observations on a single subject [3].

As a result of comparing the methods, it can be concluded that the results are "compatible" when the degree of agreement between the new method and the standard method is good. In this study, the ratio of the difference between the paired measurements to the mean was defined as the "relative error (RE)". If the number of measurements with a relative error of $\pm 20\%$ or less is within 75% of the total number of measurements, it was considered "compatible" [10]. The significance level was set at $P < 0.05$.

RESULTS

Laboratory-level precision and accuracy control tests

The data are plotted with the i-STAT 1 on the y-axis and the GEM premier 3500 as reference method on the x-axis under the laboratory condition in Fig. 2. Deming regression analysis reveals intercept=0.014 (95% confidence interval [CI]= -0.023 to 0.050) and slope=1.025 (95%CI=0.970 to 1.080). The concordance correlation coefficient is 0.979, which is considered as indicating almost perfect performance. Plotting of the residuals between the i-STAT 1 and GEM premier 3500 results against their means revealed that the i-STAT 1 readings were, on average, 0.031 mM higher. Overall, 93.5% of the observations were between the 95%CI in the Bland-Altman plot, demonstrating good agreement (Fig. 2). In addition, i-STAT 1 was “compatible” with the GEM premier 3500 because the frequency of differences between the measurements within $\pm 20\%$ of the mean was 100% (65/65, >75%).

In Fig. 3, the data are plotted with the bovine blood iCa checker on the y-axis and the GEM premier 3500 as reference method on the x-axis under the laboratory condition. Deming regression analysis reveals intercept= -0.037 (95%CI= -0.124 to 0.050) and slope=1.101 (95%CI=0.998 to 1.204). The concordance correlation coefficient is 0.959, which is considered as indicating almost perfect performance. Plotting of the residuals between bovine blood iCa checker and GEM premier 3500 results against their means revealed that bovine blood iCa checker readings were, on average, 0.038 mM higher. Overall, 91.9% of the observations were between the 95%CI in the Bland-Altman plot, demonstrating good agreement (Fig. 3). In addition, bovine blood iCa checker was “compatible” with the GEM premier 3500 because the frequency of differences between the measurements within $\pm 20\%$ of the mean was 90.8% (59/65, >75%).

In Fig. 4, the data are plotted with the bovine blood iCa checker on the y-axis and the i-STAT 1 as reference method on the x-axis under the laboratory condition. Deming regression analysis reveals intercept= -0.050 (95%CI= -0.128 to 0.028) and slope=1.073 (95%CI=0.980 to 1.166). The concordance correlation coefficient is 0.971, which is considered as indicating almost perfect performance. Plotting of the residuals between the bovine blood iCa checker and i-STAT 1 results against their means revealed that the bovine blood iCa checker results were, 0.007 mM higher on average, respectively. Overall, 91.9% of the observations were between the 95% CI in the Bland-Altman plot, demonstrating good agreement (Fig. 4). In addition, bovine blood iCa checker was “compatible” with the i-STAT 1 because the frequency of differences between the measurements within $\pm 20\%$ of the mean was 92.3% (60/60, >75%).

The clinical trials carried out used cattle with or without hypocalcemia in the bovine farm

In Fig. 5, the data are plotted with the bovine blood iCa checker on the y-axis and the i-STAT 1 as reference method on the x-axis in the field condition. Deming regression analysis reveals intercept= -0.100 (95%CI= -0.325 to 0.125) and slope=1.044 (95%CI=0.827 to 1.262). Therefore, it was suggested that there was no proportional bias between i-STAT 1 and bovine blood iCa checker in field environment condition, as the 95% CI for the intercept and slope contained 0 and 1, respectively. The concordance correlation coefficient is 0.884, which is considered as indicating almost perfect performance. Plotting of the residuals between the bovine blood iCa checker and i-STAT 1 results against their means revealed that the bovine blood iCa checker readings were, on average, -0.053 mM lower. Overall, 94.4% of the observations were between the 95%CI in the Bland-Altman plot, demonstrating good agreement (Fig. 5). In addition, bovine blood iCa checker was “compatible” with the i-STAT 1 because the frequency of differences between the measurements within $\pm 20\%$ of the mean was 100% (36/36, >75%).

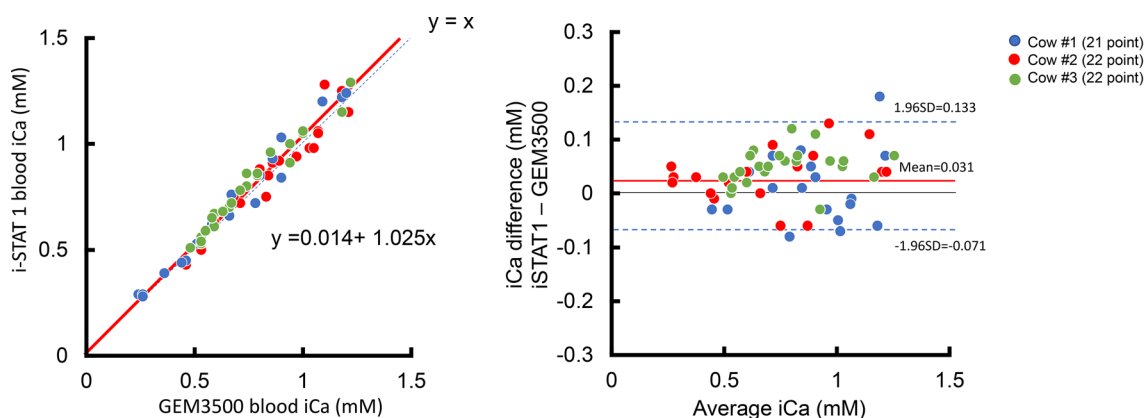


Fig. 2. Deming regression (A) and Bland-Altman plot (B) of whole blood ionized calcium (iCa) concentrations among the i-STAT 1 and laboratory benchtop blood gas analyzer GEM premier 3500 under the laboratory condition. Sixty-two paired-point whole blood samples were obtained from three cows with hypocalcemia experimentally induced by Na₂-EDTA infusion. A: The dashed and solid line represents the line of identity ($y=x$) and the regression line with intercept=0.014 (95% confidence limits [95%CI]: -0.023 to 0.050) and slope=1.025 (95%CI: 0.970 to 1.080), respectively. B: The solid line represents the mean difference between the 2 methods and the 95%CI are indicated by the dashed lines.

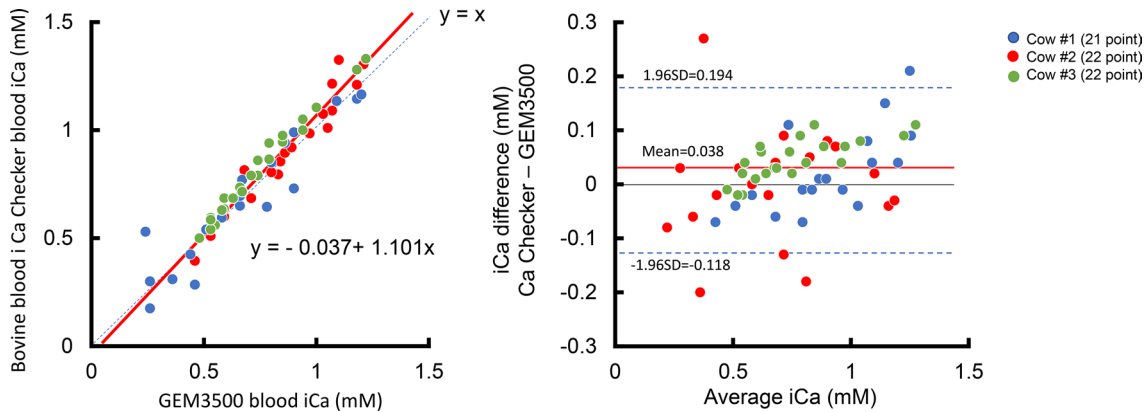


Fig. 3. Deming regression (A) and Bland-Altman plot (B) of whole blood ionized calcium (iCa) concentrations among the bovine blood iCa checker and laboratory benchtop blood gas analyzer GEM premier 3500 under the laboratory condition. Sixty-two paired-point whole blood samples were obtained from three cows with hypocalcemia experimentally induced by $\text{Na}_2\text{-EDTA}$ infusion. A: The dashed and solid line represents the line of identity ($y=x$) and the regression line with intercept= -0.037 (95% confidence interval [95%CI]: -0.124 to 0.050) and slope= 1.101 (95%CI: 0.998 to 1.204), respectively under the laboratory condition. B: The solid line represents the mean difference between the 2 methods and the 95%CI are indicated by the dashed lines.

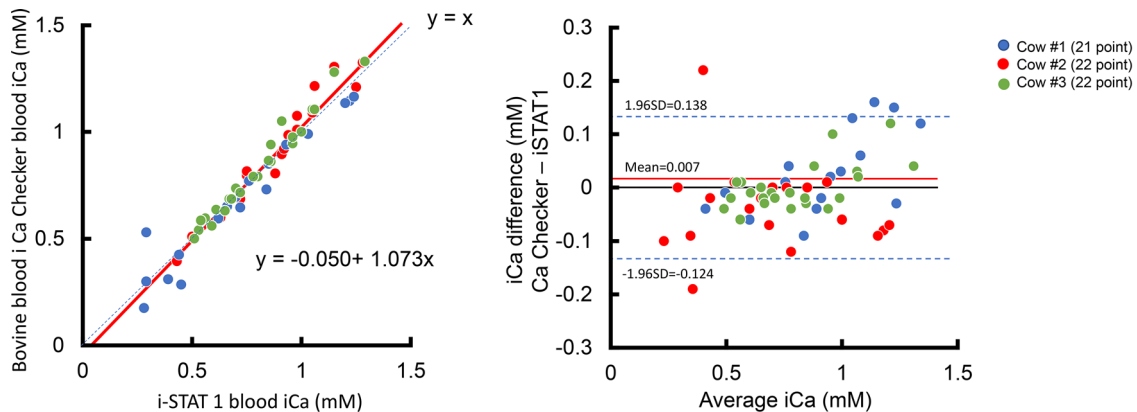


Fig. 4. Deming regression (A) and Bland-Altman plot (B) of whole blood ionized calcium (iCa) concentrations among the bovine blood iCa checker and the i-STAT 1 under the laboratory condition. Sixty-two paired-point whole blood samples were obtained from three cows with hypocalcemia experimentally induced by $\text{Na}_2\text{-EDTA}$ infusion. A: The dashed and solid line represents the line of identity ($y=x$) and the regression line with intercept= -0.050 (95% confidence interval [95%CI]: -0.128 to 0.028) and slope= 1.073 (95%CI: 0.980 to 1.166), respectively. B: The solid line represents the mean difference between the 2 methods and the 95%CI are indicated by the dashed lines.

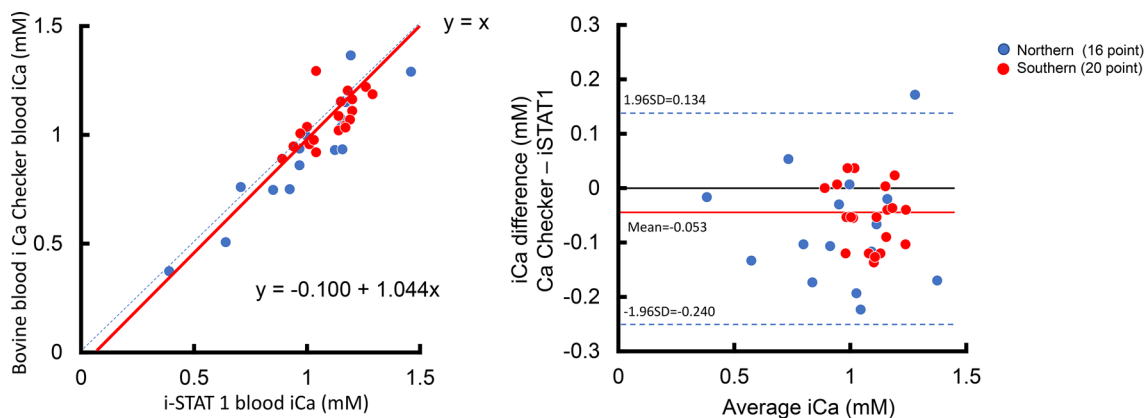


Fig. 5. Deming regression (A) and Bland-Altman plot (B) of whole blood ionized calcium (iCa) concentrations among the bovine blood iCa checker and the i-STAT 1 under the on-farm condition. Thirty-six paired-point whole blood samples were obtained from each cow in commercial farm. A: The dashed and solid line represents the line of identity ($y=x$) and the regression line with intercept= -0.100 (95% confidence interval [95%CI]: -0.325 to 0.125) and slope= 1.044 (95%CI: 0.827 to 1.262), respectively. B: The solid line represents the mean difference between the 2 methods and the 95%CI are indicated by the dashed lines.

DISCUSSION

This series of studies that consisted of laboratory-level precision and accuracy control tests, and clinical trials were to compare the commercially available ion-selective electrode handheld iCa meter (bovine blood iCa checker) with the benchtop blood gas analyzer GEM premier 3500 and handheld analyzer i-STAT 1. In addition, the iCa measurements by the bovine blood iCa checker in the field were markedly highly correlated and homologous with those by the i-STAT 1 regardless of ambient temperature. Based on these findings, the bovine blood iCa checker may be applied as a simplified system to measure the iCa concentration in bovine whole blood.

The concentration of iCa in blood preanalytical may be affected by pH changes of the sample, calcium binding by heparin, and dilution by the anticoagulant solution [6]. The iCa binding by heparin can be minimized by a final concentration of heparin sodium solution of 15 IU/ml blood or less [6]. The main causes of pH changes *in vitro* are loss of bicarbonate by non-anaerobic handling and formation of lactic acid. Therefore, pH changes of the sample were avoided by anaerobic handling, rolling to mixing sample by hand and minimizing the delay before measurement (≤ 15 min) [5]. Therefore, whole blood was anaerobically carefully collected using a syringe containing 50 IU sodium heparin solution (final concentration of 10 IU/ml -blood) and subjected to measurement for iCa concentration within 5 min in the present study [5, 6].

Whole blood iCa concentrations measured by the GEM premier 3500 blood gas analyzer were considered the reference values in laboratory-level precision and accuracy control tests. Deming regression model analysis was also performed in this study [16]. Whereas the ordinary linear regression method assumes that only the Y measurements are associated with random measurement errors, the Deming method takes measurement errors for both methods into account [8]. The standard deviation (SD) of the residual error of regression calculated as a measure of the random error in the regression line due to all correlation coefficients was markedly influenced by the range of the data [9]. In 1983 Altman and Bland [2] proposed an alternative analysis, based on the quantification of the agreement between two quantitative measurements by studying the mean difference and constructing limits of agreement. In this study, the Bland-Altman plot were used to assess the residuals between results from the two devices and those of the laboratory tests.

Even if the measurement accuracy and sensitivity of the benchtop type blood gas analyzer such as GEM premier 3500 are excellent, it is not suitable using field condition since not possible to measure the blood gases immediately after sampling from cow. Therefore, before conducting this series of studies, it was verified with GME3500 as reference device whether handheld analyzer i-STAT 1 was suitable as a reference device for clinical trials. As a result, it is suggested that the iCa measurement ability of i-STAT 1 is markedly highly correlated and homologous when the GEM premier 3500 is used as the reference method. This study confirmed previous reports that iCa concentrations in bovine blood measured by the i-STAT 1 agree with those by the radiometer as the reference method, and that the i-STAT 1 can predict hypocalcemia with a high sensitivity [30]. Therefore, the i-STAT 1 is a useful POC instrument to measure the iCa concentration in cows, consistent with previous studies that it is suitable for measuring the iCa concentration in whole blood [31].

Results from laboratory-level precision study, the iCa measurement ability of the bovine blood iCa checker is markedly highly correlated and homologous when the GEM premier 3500 and i-STAT 1 are used as the reference methods. Therefore, the bovine blood iCa checker is a useful POC instrument to measure the iCa concentration, similar to i-STAT 1. Although the bovine blood iCa checker was satisfactory in the quality control test using an experimental hypocalcemic cow model in the laboratory environment, it is necessary to investigate whether it can be clinically applied in the field environment.

The i-STAT 1 is an excellent POC for use in small animal [28], equine [27], and cattle practice [4, 17, 24, 30]. However, due to its structure, a temperature environment between 17°C and 30°C (62.6–86.0°F) is required. Unlike the i-STAT 1, no temperature conditions must be met to use the bovine blood iCa checker because it is not equipped with a measurement heater. This is highly advantageous for bovine practice since measurement is possible unless the sample freezes.

It is well known that the i-STAT 1 is superior for blood gas and biochemistry analysis when performing screening tests [4, 17, 24, 30]. The bovine blood iCa checker simplifies blood iCa concentration measurement as a cow-side test regardless of the ambient temperature. Therefore, depending on the clinical situation, bovine practitioners can determine the treatment method for milk fever and hypocalcemia at the farm by using the either in i-STAT 1 that can simultaneously measure other parameters or bovine blood Ca checker that can easily measure only iCa concentration.

In the clinical trial, pairs of observations using the two methods are independent, Bland-Altman plot analysis were very simple and straightforward due to different subjects. However, laboratory-level precision trials obtained repeated pairs of measurements on the same subject. Therefore, Bland-Altman analysis were performed using an above commercial statistical software to analyzing such clustered observations, both when the underlying quantity is assumed to be changing and when it is not [3, 13, 32]. A repeated Bland-Adams approach is to average the repeated measurements for each subject and use only number of subject's pairs of means to calculate the 95% limits. That analysis would be expected to give limits that are too narrow.

The clinical trials conducted in the farm environment cow-side also revealed that the high accuracy in blood iCa measurement of the bovine blood iCa checker was similar to that of the iSTAT1 as a reference device, as in the laboratory experiment using the experimental hypocalcemic cow model.

In conclusion, the bovine blood iCa checker and i-STAT 1 are useful POC analyzers that can be used safely, rapidly, and simply in animal hospitals, ambulatory clinics, and dairy farms to evaluate the blood iCa concentration in cows. Moreover, bovine practitioners can rapidly decide the treatment method for milk fever and hypocalcemia beside the cow because the bovine blood Ca checker can measure the iCa concentration regardless of ambient temperature.

CONFLICT OF INTEREST DECLARATION. None of the authors have any financial or personal relationships that may have inappropriately influenced or biased the content of the paper. In addition, authors declare no off-label use of antimicrobials and human ethics approval was not needed for this study.

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