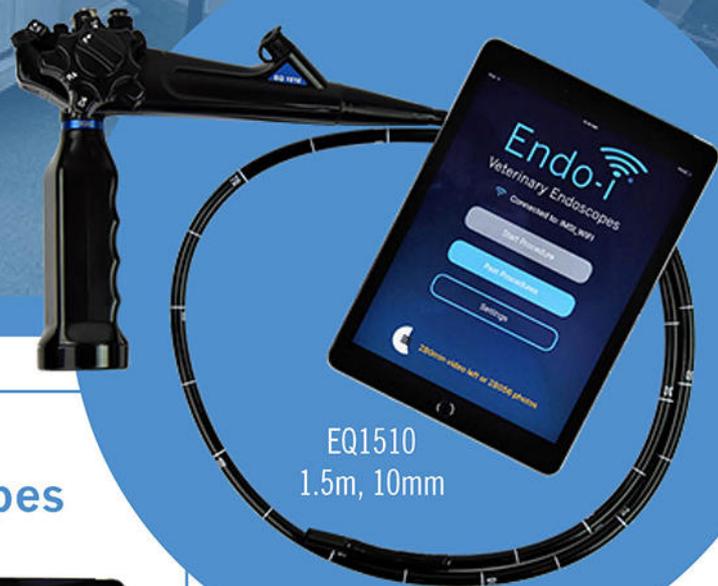


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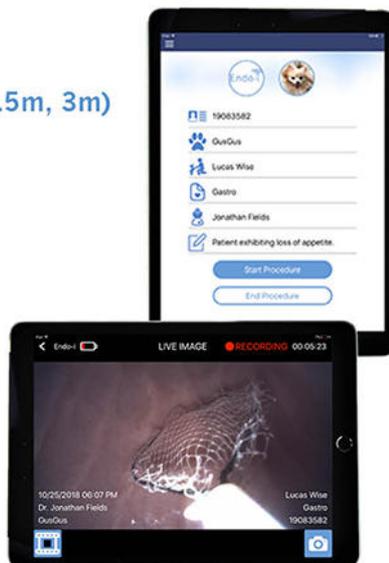
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Effects of pH and the plasma or serum concentrations of total calcium, chloride, magnesium, L-lactate, and albumin on the plasma ionized calcium concentration in calves

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Abstract

Background: The plasma ionized calcium concentration (cCa^{2+}) represents the biologically active form of calcium and is the preferred method for evaluating calcium status in animals. Different pH-corrective equations have been developed for human plasma, but the validity of the equations for bovine plasma is unknown.

Hypothesis: We hypothesized that pH-corrective equations for bovine plasma would be similar to those used for human plasma; cCa^{2+} was dependent on the plasma concentrations of total calcium ($cTCa$), chloride (cCl), L-lactate ($cLactate$), and albumin ($cAlbumin$); and the in vitro and in vivo cCa^{2+} -pH relationships would differ.

Animals: Ten healthy calves (in vitro study), 1426 critically ill calves.

Methods: The in vitro plasma $\log_{10}(cCa^{2+})$ -pH relationship was determined by CO_2 tonometry of 465 plasma samples. Plasma cCl was altered by equivolume dilution of plasma with 3 electrolyte solutions of different cCl . The in vivo plasma cCa^{2+} -pH relationship was investigated and validated using clinicopathologic data extracted from the medical records of 950 (model development) and 476 (model validation) critically ill calves.

Results: pH-corrective equations for bovine plasma were similar to those used for human plasma. Plasma cCa^{2+} increased in vitro with increases in plasma cCl . Plasma cCa^{2+} in critically ill calves was associated with plasma $cTCa$, blood pH, plasma cCl , serum cMg , and cL -lactate ($R^2 = 0.69$) but not plasma $cAlbumin$.

Conclusions and Clinical Importance: Calculation of cCa^{2+} from $cTCa$ in calf plasma or serum requires adjustment for at least pH and cCl when 1 or both are outside the reference range.

KEYWORDS

hyperchloremia, hyperlactatemia, ionized calcium, ion-selective potentiometry

Abbreviations: $cAlbumin$, albumin concentration; cCa^{2+} , ionized calcium concentration; cCl , chloride concentration; $cGlobulin$, globulin concentration; cK , potassium concentration; $cLactate$, lactate concentration; cMg , magnesium concentration; cNa , sodium concentration; $cPhosphorus$, phosphorus concentration; $cTCa$, total calcium concentration; $cTotal\ protein$, total protein concentration.

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1 | INTRODUCTION

The calcium fractions in bovine plasma are in equilibrium and exist in 3 forms: free (43%–57% of total), bound to proteins in a salt-type manner (35%–49%), and complexed to anions in plasma

(approximately 8%). The plasma ionized (free) calcium fraction is the biologically active form of calcium and therefore is the preferred analyte for calcium measurement. The plasma ionized calcium concentration (cCa^{2+} ; where c indicates molar concentration) is primarily dependent on the total calcium concentration ($cTCa$),¹ with $cTCa$ explaining 64% to 86% of the variation in cCa^{2+} in plasma or serum from adult cattle.²⁻⁴ The cCa^{2+} is also dependent on pH, plasma concentrations of albumin ($cAlbumin$), globulin ($cGlobulin$), chloride (cCl), and L-lactate (cL -lactate), as well as the temperature and ionic strength and therefore the plasma concentration of sodium.⁵⁻⁹

Apart from $cTCa$, pH is believed to have the largest effect on the cCa^{2+} of calf plasma¹⁰ and cow plasma,¹¹ with $\log_{10}cCa^{2+}$ being negatively and linearly associated with plasma pH. Correction of the measured ionized calcium concentration to pH = 7.40 ($cCa^{2+}_{7.40}$) has been used in experimental studies in cattle to assist in interpretation of measured results relative to a reference range.^{3,4,12-14} The formula used in some analyzers for pH correction of measured cCa^{2+} (cCa^{2+}_m) in blood, plasma, or serum is derived from in vitro CO_2 tonometry studies using human serum and the resultant buffer capacity line slope S ($\Delta\log_{10}cCa^{2+}/\Delta pH$), whereby,

$$\log_{10}cCa^{2+}_{7.40} = \log_{10}cCa^{2+}_m + S \times (7.40 - pH_m) \quad (1)$$

where pH_m in Equation 1 is the measured pH.¹⁵⁻¹⁷ The experimentally determined value for S in human serum with a total protein concentration of 70 g/L is -0.23 or -0.24 .¹⁵⁻¹⁸ The effect of pH is clinically relevant in that there is a 5.3% increase in cCa^{2+} for every 0.1 unit decrease in in vitro pH within the physiologic range of pH.¹⁸ The pH-corrective equation was designed to account for the effect of increased plasma pH caused by the escape of CO_2 from samples that were not anaerobically collected or maintained. The International Federation of Clinical Chemistry recommended in 1991 that Equation 1 be applied to human serum or blood samples with normal albumin and total protein concentrations over a pH range of 7.2 to 7.6 using a value of S ranging from -0.16 to -0.24 .¹⁷ The validity of pH adjustment equations for plasma from species other than humans is unknown, and our first hypothesis was that the experimentally determined value for S in Equation 1, when applied to calf plasma, would be similar to -0.23 or -0.24 because bovine and human plasma have similar values for net protein charge and the effective dissociation constant for plasma proteins.¹⁹

The experimentally determined value for S in vitro is higher than that in vivo because the magnitude of S is proportional to $cAlbumin$.^{15,16} Rapid equilibration of pH and calcium between plasma and interstitial fluid means that the "apparent" in vivo plasma $cAlbumin$ is lower than the measured in vitro plasma $cAlbumin$.¹⁶ Our second hypothesis therefore was that the in vivo value for S in critically ill calves would be less than the experimentally determined in vitro values for calf plasma. A related hypothesis was that calf plasma with decreased $cAlbumin$ would have a lower in vitro value for S than calf plasma with the plasma $cAlbumin$ within the reference range.

Bovine and human albumins have a net negative charge at physiologic pH,^{19,20} and it is likely therefore that albumin binds some cations

in a salt-type manner through electrostatic attraction. Quantitatively important amounts of Ca and Mg are bound to human albumin at physiologic pH,⁹ but Na and K do not appear to be bound to human albumin²¹ and are assumed not to bind to bovine albumin. Plasma $cAlbumin$ and $cGlobulin$ therefore are likely to influence cCa^{2+} in calf plasma¹⁰ and cow plasma^{11,22} by electrostatic binding of ionized Ca, but calcium binding to proteins in cattle appears to occur to a lesser extent than that for plasma from other species.²² Our fourth hypothesis therefore was that the slope of the in vitro $\log_{10}cCa^{2+}$ -pH relationship would be influenced by $cAlbumin$ in calf plasma but to a lesser extent than the effect of $cAlbumin$ on the slope value in human plasma.²³

Chloride ions are bound in a salt-type manner to positively charged guanidinium and ϵ -amino groups in albumin despite the net negative charge of albumin at physiologic pH.²⁴ Three chloride ions are electrostatically bound to each bovine albumin molecule at pH = 7.40.²⁵ An overlooked physicochemical phenomenon is that an increase in bovine serum cCl increases the number of chloride ions bound to bovine albumin.²⁵ Chloride binding to albumin in human, dog, and rat serum displaces calcium from adjacent electrostatic binding sites, thereby increasing serum cCa^{2+} .^{8,26-28} Our fifth hypothesis therefore was that hyperchloremia would increase cCa^{2+} in calf plasma.

Based on the 5 hypotheses, the primary objective of our study was to use CO_2 tonometry to characterize the in vitro relationship between $\log_{10}cCa^{2+}$ and pH for plasma from healthy calves. Additional objectives were to determine whether the plasma $cAlbumin$ influences the in vitro $\log_{10}cCa^{2+}$ -pH relationship, verify that hyperchloremia increases cCa^{2+} in calf plasma as in other species, and characterize the in vivo relationship between cCa^{2+} and selected variables related to protein binding of Ca (pH, cCl) and complexing of Ca (L-lactate, uremic anions) in plasma from critically ill calves.

2 | MATERIALS AND METHODS

2.1 | In vitro study using CO_2 tonometry

Twenty milliliters of venous blood was collected into 10 mL lithium-heparin vacutainer tubes containing 150 international units (IU) of lithium heparin from the jugular vein of 5 female and 5 male healthy Holstein-Friesian calves, 4 to 55 days of age. This produced a calculated heparin concentration of 15 and 7.5 IU/mL for undiluted and equivolume-diluted plasma samples, respectively; heparin concentrations <15 IU/mL are recommended to minimize calcium binding to heparin.¹⁷ Plasma was harvested within 30 minutes of collection by centrifugation and stored in polypropylene cryogenic vials at $-70^\circ C$ for up to 2 months. Plasma was thawed at room temperature immediately before CO_2 tonometry, and equivolume dilution with solutions of different cCl was performed. Plasma samples were tonometered (Model IL 235; Instrumentation Laboratory, Lexington, Massachusetts) for 20 minutes at $37^\circ C$ over a pCO_2 range of 19 to 182 mm Hg and a corresponding pH range of 7.70 to 6.90 using a variable mixture of humidified 20% CO_2 and 100% O_2 . This pH range was selected because it lies within the linear portion of the calcium-protein dissociation curve for human plasma.⁷ Plasma cCl was altered before tonometry by

TABLE 1 Calculated composition of equivolume diluent for plasma samples and results of in vitro CO₂ tonometry of the equivolume diluted plasma samples from 10 healthy calves

Factor	Low cCl ⁻ (n = 102)	Middle cCl ⁻ (n = 108)	High cCl ⁻ (n = 113)
Calculated composition of equivolume diluent			
cCl (mmol/L)	95	120	145
cHCO ₃ (mmol/L)	70	44	20
cPhosphate (mmol/L)	2	2	2
cSO ₄ (mmol/L)	1.25	1.25	1.25
cNa (mmol/L)	156	154	154
cK (mmol/L)	5.0	5.0	5.0
cTCa (mmol/L)	2.5	2.5	2.5
cMg (mmol/L)	2.0	2.0	2.0
In vitro CO ₂ tonometry of equivolume diluted plasma			
cCl (mmol/L)	97.0 ± 1.8	110.5 ± 1.5	123.4 ± 1.3
cNa (mmol/L)	147.8 ± 1.2	149.0 ± 1.1	149.6 ± 1.1
cK (mmol/L)	2.2 ± 0.2	2.3 ± 0.2	2.2 ± 0.2
Pco ₂ (mm Hg)	96 ± 32	96 ± 37	77 ± 38
pH	7.37 ± 0.15	7.26 ± 0.17	7.21 ± 0.22
cCa ²⁺ (mmol/L)	0.71 ± 0.05	0.78 ± 0.06	0.82 ± 0.06
S	-0.151 (-0.162 to -0.139)	-0.133 (-0.147 to -0.120)	-0.116 (-0.122 to -0.111)
R ²	0.93	0.88	0.96

The primary manipulated factor in the diluent was cCl. Tonometered data is presented as mean ± SD or mean and 95% CI in parentheses. S = slope of the log₁₀cCa²⁺-pH relationship during tonometry.

equivolume dilution of plasma with 3 electrolyte solutions of different cCl (Table 1); pCO₂ tonometry therefore was performed at 4 different cCl and 2 different values for cAlbumin and cGlobulin for each calf.

The jugular venous blood sample and all tonometered plasma samples were analyzed in duplicate using a blood/plasma gas analyzer and standard methodology (Statprofile 9+; NOVA Biomedical, Canada LTD, Mississauga, Ontario, Canada): pH (hydrogen ion selective glass membrane), cCa²⁺, cNa, cK, and cCl (ion-selective potentiometry), and cL-lactate (lactate oxidase). The instrument was calibrated every 2-6 hours using a 2-point calibration to measure the electrode slope and verify electrode performance. An untonometered plasma sample was analyzed in an autoanalyzer (Dacos multianalyzer; Coulter Electronics, Hialeah, Florida) in duplicate for determination of cAlbumin (bromocresol green), cTotal protein (biuret), total magnesium concentration (cMg; calmagite), and phosphate (cPhosphate; ammonium molybdate) concentrations. Plasma globulin concentration (cGlobulin) was calculated as the difference between plasma cTotal protein and cAlbumin. Partial results of CO₂ tonometry of the undiluted plasma samples have been reported elsewhere.¹⁹ Based on the measured analyte concentrations, the plasma D-lactate concentration (cD-lactate) was estimated to be <1.0 mmol/L and therefore quantitatively unimportant.

2.2 | In vivo study of critically ill calves

The medical records of 10 060 critically ill calves up to an age of 21 days admitted to the Clinic for Ruminants, LMU Munich, between November 1997 and March 2016 were reviewed. The population in

the study reported here, comprising 1553 critically ill calves admitted from April 2005 to January 2008, was a subset of this much larger data set.²⁹ Data were extracted from the medical records of the 1553 calves because during this period cTCa was measured as part of a routine biochemistry panel, and whole-blood pH, pCO₂, pO₂, and iCa, Na, K, and Cl concentrations were obtained using the same analyzer. Results for selected jugular venous blood, plasma, and serum analytes were extracted from the medical record, including blood pH (hydrogen ion selective glass membrane), and cCa²⁺, cNa, cK, and cCl (ion-selective potentiometry) measured using a blood pH, gas, and electrolyte analyzer (Rapidlab 865 blood gas analyzer; Bayer Vital GmbH, Fernwald, Germany). Blood pH and pCO₂ were corrected for rectal temperature using standard algorithms.³⁰ Actual bicarbonate concentration (cHCO₃) was calculated by using the Henderson-Hasselbalch equation with measured blood pH and pCO₂ at 37°C: cHCO₃ = S × pCO₂ × 10^(pH-pK₁'). Values used for the negative logarithm of the dissociation constant of carbonic acid (pK₁') and solubility of carbon dioxide (S) for plasma at 37°C were 6.105 and 0.0307 mmol/L per mm Hg, respectively. An automated analyzer (Hitachi 911; Roche Diagnostics, Indianapolis, Indiana) was used for biochemical analysis. Plasma cL-lactate and cD-lactate were determined photometrically by means of enzymatic methods using L- and D-lactate dehydrogenase, respectively.^{31,32} Serum samples were used to measure cAlbumin (bromocresol green), cTotal protein (biuret), cPhosphate (ammonium molybdate), cMg (xylydyl blue), cUrea (urease), and cCreatinine (picric acid).

2.3 | Statistical analysis

Software programs (PROC MIXED, PROC REG; SAS 9.4, SAS Inc, Cary, North Carolina; MedCalc Statistical Software version 15.11.4; MedCalc Software bvba, Ostend, Belgium) were used for statistical analyses and variables with an apparently normal distribution based on the Shapiro-Wilk test are presented as mean \pm SD. Non-normally distributed variables are presented as median and range. Values of $P < .01$ were considered statistically significant because of the relatively large data set and number of comparisons. The cCa^{2+} (in mmol/L) was expressed as a percentage of $cTCa$ (in mmol/L), such that ionized calcium percentage = $(cCa^{2+} \times 100)/cTCa$. The serum globulin concentration ($cGlobulin$) was calculated as the difference between $cTotal$ protein and $cAlbumin$.

The in vitro relationship between plasma $\log cCa^{2+}$ and pH was investigated using mixed models analysis with calf as the subject, an unstructured covariance matrix (selected because it provided the lowest Akaike Information Criterion value), and a random intercept. This approach fitted a similar slope to data from all calves but permitted a different intercept value for each calf. The 95% confidence interval (CI) for the coefficient estimate was calculated for significant predictors. The accuracy of the estimated values was evaluated by comparing actual versus predicted values for the $\log cCa^{2+}$ -pH relationship and examination of residual plots to identify statistical outliers.

Analysis of the in vivo data set was confined to calves with blood pH of 6.90 to 7.70 to ensure consistency with the results of in vitro tonometry. The data set was randomly separated into a model building set (67% of medical records) and a validation data set (33% of medical records) using a random number generator (Excel; Microsoft, Seattle, Washington). Univariate analysis using linear regression was performed to characterize the relationship between $\log_{10}cCa^{2+}$ or cCa^{2+} and predictor variables of interest; $\log_{10}cCa^{2+}$ and cCa^{2+} were evaluated separately to facilitate comparisons to other studies.

Derivation of a mathematical equation for cCa^{2+} that was consistent with the algebraic relationship in Equation 1 would facilitate interpretation of multivariable regression equation slopes with cCa^{2+} as the dependent variable. Equation 1, therefore, was algebraically manipulated by raising both sides of the equation to the power of 10 to provide the following equivalent expression:

$$cCa^{2+}_{7.40} = cCa^{2+}_m \times 10^{S \times (7.40 - pH_m)} \quad (2)$$

Blood gas and pH analyzers that use Equation 2 for pH correction typically use a value for S of -0.178 ,³³ which is thought to reflect the in vivo buffer capacity line slope in humans.³⁴ Some analyzers use the following equivalent expression to Equation 1:

$$cCa^{2+}_{7.40} = cCa^{2+}_m \times (1 + B \times [7.40 - pH_m]), \quad (3)$$

where $B = \ln(10) \times S = 2.303 \times S$,¹⁸ producing values for B of -0.50 or -0.53 when $S = -0.23$ or -0.24 , respectively.^{7,18,23} In other words, when $B = -0.53$, there is a 5.3% decrease in cCa^{2+} per 0.1 increase in pH.¹⁸

A stepwise forward multivariate regression model was developed to determine the relationship between cCa^{2+} and selected independent variables of interest. Variables were selected for inclusion in the stepwise regression procedure because they were biologically relevant based on the documented evidence of calcium binding in plasma or serum or they had the potential to alter calcium binding to albumin and globulin at physiologic pH. Moreover, to minimize the effects of collinearity and ensure an appropriately low variance inflation factor for individual values, when 2 variables were closely correlated ($r_s > 0.60$), only the variable that had the highest r_s for cCa^{2+} was entered into the model.³⁵ The relative importance of the included variables was assessed by the order of entry into the model as well as by the change of the model R^2 value (ΔR^2). Residual plots of each multivariable model and partial models and normality plots were examined to confirm an approximately normal distribution of residuals, absence of outliers, linearity of the response, and absence of heteroscedasticity.³⁵ Interaction terms were not investigated for each significant predictor in the multivariate model because interaction terms made it difficult to interpret coefficient values.³⁵

Corrective equations based on the final stepwise regression model were developed using the multivariate equations for the first 1, 2, and 3 variables to enter the stepwise regression procedure to calculate cCa^{2+} from the validation data set. The calculated values for cCa^{2+} then were compared to the measured cCa^{2+} using Passing-Bablok regression and Bland-Altman plots.

3 | RESULTS

3.1 | In vitro study using CO₂ tonometry

A total of 465 CO₂ tonometered plasma samples were analyzed from the 10 calves; 142 from undiluted calf plasma, and 102, 108, and 113 from diluted plasma with low (97.0 ± 1.8 mmol/L), mid (110.5 ± 1.5 mmol/L), and high (123.4 ± 1.3 mmol/L) cCl, respectively (Table 1). Mixed models analysis was applied using the measured pH and $\log_{10}cCa^{2+}$ values from 4 to 14 data points for each calf obtained during CO₂ tonometry for the 4 groups. The mean values were 1.20 ± 0.15 mmol/L (cCa^{2+}), 31.2 ± 2.5 g/L ($cAlbumin$), 56.4 ± 3.5 g/L ($cTotal$ protein), 25.2 ± 4.7 g/L ($cGlobulin$), 139.3 ± 2.2 mmol/L (cNa), 4.5 ± 0.4 mmol/L (cK), 1.0 ± 0.4 mmol/L (cMg), 101.1 ± 2.3 mmol/L (cCl), 1.9 ± 1.0 mmol/L (cL -lactate), and 2.6 ± 0.2 mmol/L ($cPhosphate$).

A negative linear relationship was found between $\log_{10}cCa^{2+}$ and pH for undiluted plasma (Figure 1). The coefficient for the slope ($S = \Delta \log_{10}cCa^{2+} / \Delta pH$) of the univariate linear regression equation relating $\log_{10}cCa^{2+}$ to pH was -0.227 (95% CI, -0.235 to -0.220). The value for S in undiluted plasma was equivalent to -0.0072 (95% CI, -0.0075 to -0.0070) for each gram of albumin per liter and -0.0040 (95% CI, -0.0042 to -0.0038) for each gram of total protein per liter.

Equivolume dilution of plasma samples produced calculated mean values for $cAlbumin$ of 15.6 g/L and $cTotal$ protein of 28.7 g/L. The estimated slope (S) for the $\log_{10}cCa^{2+}$ -pH relationship varied directly and linearly with cCl (Table 1). Linear regression analysis of the

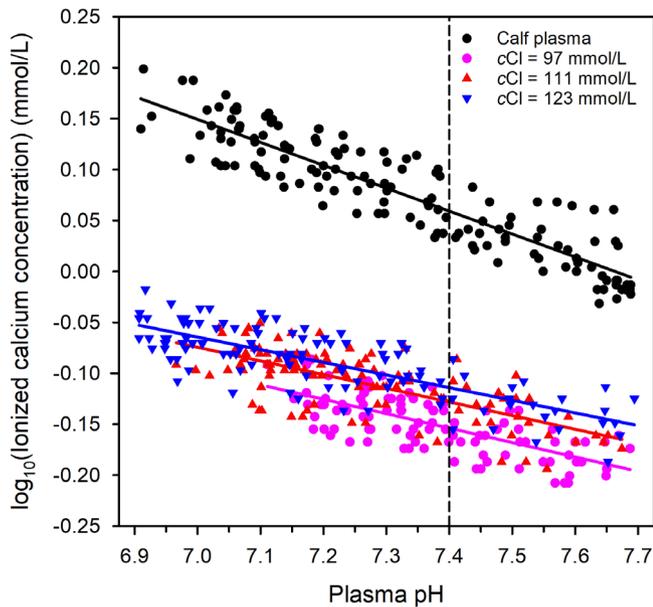


FIGURE 1 In vitro relationship between the ionized calcium concentration (logarithmic scale) and plasma pH in 10 healthy calves. Plasma pH was varied in undiluted plasma using CO₂ tonometry (filled circles) and equivolume dilution of plasma with a solution of low cCl (pink circles), medium cCl (orange triangles), or high cCl (inverted blue triangles) followed by CO₂ tonometry. The vertical dashed line indicates pH = 7.40. Note that cCa²⁺ increases with cCl and the slopes of the equivolume diluted plasma samples become steeper (more negative) with increased cCl

relationship between S and cCl for plasma samples obtained by equivolume dilution produced the following equation: $S = -0.277 + 0.0013 \times cCl$ ($R^2 = 0.999$). Application of this equation provided a calculated value for S of -0.146 for equivolume diluted plasma at $cCl = 101.1$ mmol/L (mean cCl for in vitro tonometry of undiluted plasma). The calculated value of S (-0.146) in equivolume diluted plasma was slightly more than half of the calculated value for undiluted plasma (-0.227).

Mixed models analysis was performed using 323 data points from the 3 equivolume dilution groups containing low, mid, and high cCl. The fixed effects model based on the 2 manipulated independent variables (pH, cCl) was $cCa^{2+} = 2.18 - (0.231 \times pH) + (0.00246 \times cCl)$.

3.2 | In vivo study of critically ill calves

Sixty-seven of the 1553 calves had blood pH <6.90, and data from these calves were excluded from analysis. Univariate and multivariate regression analysis identified that data points from an additional 60 calves were statistical outliers, based on residual values >+3.5 or <-3.5. Consequently, data from 1426 calves were available for analysis; 950 for model development, and 476 for model validation. Inclusion of all data from the 60 excluded medical records did not alter any of the regression models regarding statistical significance but slightly increased the SE for most independent variables and decreased R^2 by <0.03.

The median and range of selected variables are presented in Table 2, and the median ionized calcium percentage was 47%.

TABLE 2 Results of univariate regression for independent whole blood, plasma, or serum variables of interest in predicting the in vivo plasma ionized calcium concentration in 950 critically ill neonatal calves on admission to a Veterinary Teaching Hospital

Variable	Median (range)	$\Delta \log_{10} cCa^{2+} / \Delta \text{variable}$	R^2	$\Delta cCa^{2+} / \Delta \text{variable}$	R^2	P-value
Ionized calcium (mmol/L)	1.19 (0.90-1.59)	NA	NA	NA	NA	<.001
Ionized calcium (%)	47 (35-61)	NA	NA	NA	NA	<.001
Total calcium (mmol/L)	2.55 (1.67-3.54)	0.078	0.344	0.225	0.352	<.001
pH	7.31 (6.90-7.54)	-0.137	0.316	-0.395	0.321	<.001
HCO ₃ (mmol/L)	27.0 (4.5-52.0)	-0.0020	0.294	-0.0058	0.285	<.001
D-lactate (mmol/L)	0.8 (0-23.3)	0.0035	0.239	0.0095	0.219	<.001
Chloride (mmol/L)	101 (73-145)	0.0019	0.192	0.0055	0.196	<.001
Sodium (mmol/L)	135 (110-188)	0.0011	0.068	0.0033	0.071	<.001
Albumin (g/L)	27.3 (15.1-46.7)	0.0021	0.050	0.0062	0.053	<.001
Total protein (g/L)	53.1 (26.8-108.9)	0.0007	0.040	0.0022	0.048	<.001
L-lactate (mmol/L)	1.8 (0.2-19.2)	-0.0027	0.037	-0.0072	0.034	<.001
Globulin (g/L)	25.1 (9.2-84.4)	0.0006	0.021	0.0020	0.027	<.001
Total magnesium (mmol/L)	0.88 (0.45-2.28)	0.013	0.009	0.049	0.014	.002
Creatinine (mmol/L)	0.118 (0.01-1.02)	NS	NS	NS	NS	NS
Urea (mmol/L)	0.008 (0.001-0.060)	NS	NS	NS	NS	NS
Potassium (mmol/L)	4.6 (2.1-11.5)	NS	NS	NS	NS	NS
Phosphate (mmol/L)	2.6 (1.2-8.5)	NS	NS	NS	NS	NS

Abbreviations: $\Delta \log_{10} cCa^{2+} / \Delta \text{variable}$, the slope of the $\log_{10} cCa^{2+}$ -variable relationship; $\Delta cCa^{2+} / \Delta \text{variable}$, the slope of the cCa^{2+} -variable relationship; NA, not applicable; NS, not significant ($P > .01$).

TABLE 3 Results of stepwise forward multivariate regression for the prediction of in vivo plasma ionized calcium concentration in 950 critically ill neonatal calves

Order of entry	Variable	Coefficient	SE	P value	Partial R ²	Model R ²	Variance inflation factor
	Intercept	2.90	0.13	<.001
1	Total calcium (mmol/L)	0.195	0.007	<.001	0.339	0.339	1.1
2	pH	-0.328	0.016	<.001	0.190	0.529	1.6
3	Chloride (mmol/L)	0.0028	0.0002	<.001	0.096	0.625	1.2
4	Magnesium (mmol/L)	-0.086	0.009	<.001	0.048	0.672	1.6
5	L-lactate (mmol/L)	-0.0059	0.0008	<.001	0.019	0.691	1.2

The stepwise forward multivariate regression model was developed using significant ($P < .01$) whole-blood, plasma, or serum predictors identified on univariate analysis in Table 2.

Univariate analysis indicated that plasma cTCa, blood pH, plasma cHCO_3^- , cD-lactate , cCl, cL-lactate and cNa, serum cAlbumin, cGlobulin and cTotal Protein, and serum cTMg were significant univariate

predictors of $\log_{10}\text{cCa}^{2+}$ and cCa^{2+} (Table 2). Spearman's correlation coefficient indicated that cHCO_3^- and cD-lactate were associated with blood pH ($r_s = 0.86$ and 0.68 , respectively) and consequently were not

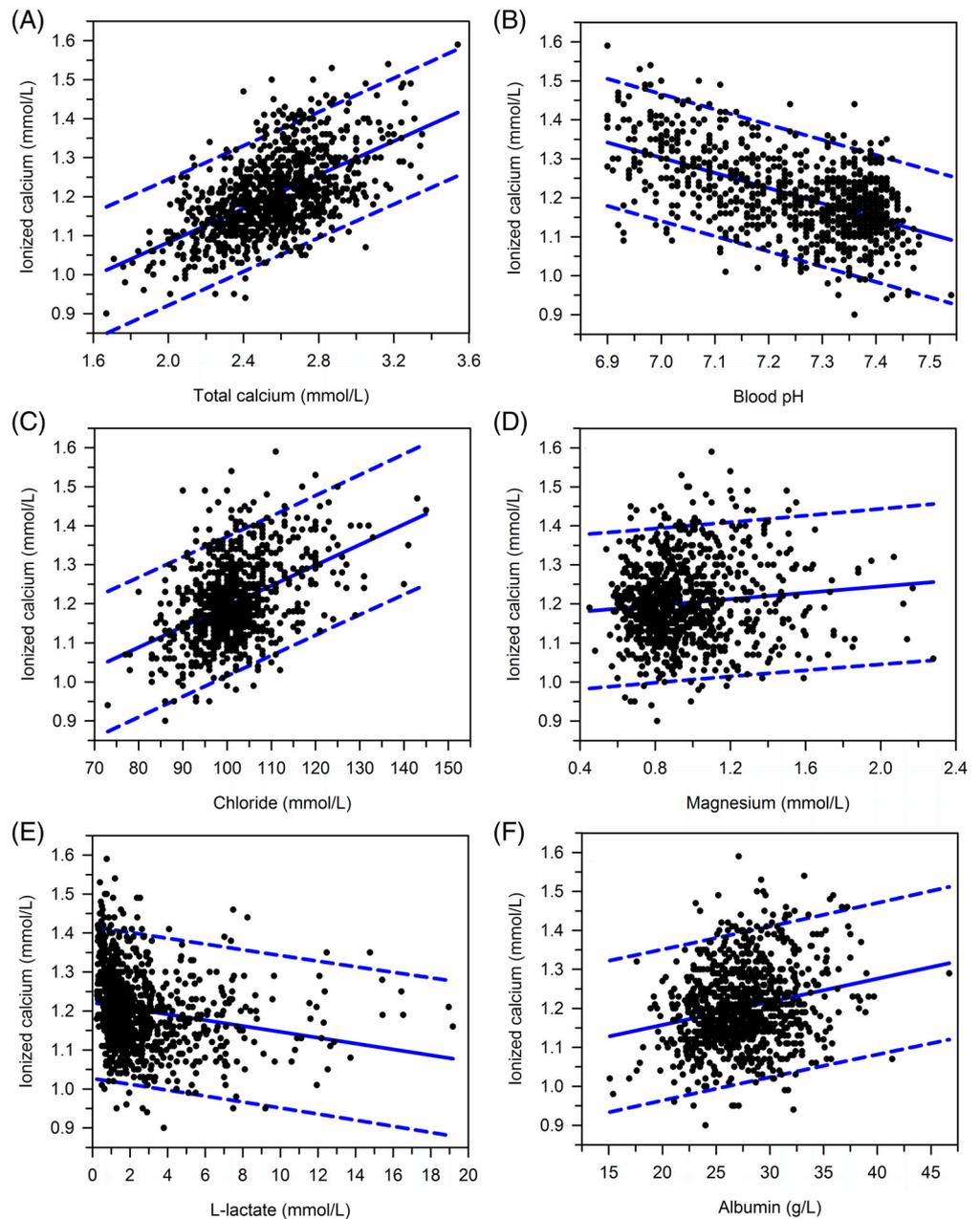


FIGURE 2 Scatterplots of variables of interest (plasma total calcium concentration, blood pH, plasma chloride concentration, serum magnesium concentration, plasma L-lactate concentration, and serum albumin concentration) depicting their linear relationship (solid blue line) with plasma ionized calcium concentration in 950 critically ill neonatal calves admitted to a veterinary teaching hospital. The dashed blue line is the 95% confidence interval for prediction

included in the stepwise linear regression procedure. Similarly, cNa was associated with cCl ($r_s = 0.62$) and consequently was not included in the stepwise regression procedure.

The final forward stepwise multivariate regression model identified 5 predictors (plasma cTCa, blood pH, plasma cCl, cMg, and cL-lactate) that explained 69% of the variation in plasma cCa^{2+} (Table 3, Figure 2). Plasma cTCa, blood pH, and plasma cCl had the highest explanatory power, accounting for 34%, 19%, and 10% of the variation in plasma cCa^{2+} , respectively. Interestingly, serum cAlbumin or cGlobulin were not identified as significant predictors of plasma cCa^{2+} on stepwise multivariate regression. Plasma cNa was not included in the multivariate model because it was associated with cCl. Replacement of cCl with cNa in the stepwise regression procedure resulted in minor changes to the order of entry into the regression procedure for cTCa, pH, cMg, cLactate, and cNa. The partial R^2 for cNa was 0.021 and the multivariate model R^2 was 0.663; both R^2 values were lower than those obtained when cCl replaced cNa in the model (partial $R^2 = 0.096$, multivariate model $R^2 = 0.691$). Moreover, cCl entered the multivariate model first when both cCl and cNa were included in the model, with variance inflation factors of 3.9 and 3.2 for cCl and cNa, respectively.

The following linear regression equations were developed for the first 1, 2, and 3 independent variables to enter the stepwise regression procedure with plasma $cCa^{2+}_{\text{calculated}}$, cTCa, and cCl measured in mmol/L:

$$cCa^{2+}_{\text{calculated}} = 0.65 + (0.216 \times cTCa) \quad (4)$$

$$cCa^{2+}_{\text{calculated}} = 3.00 + (0.172 \times cTCa) - (0.308 \times pH) \quad (5)$$

$$cCa^{2+}_{\text{calculated}} = 2.03 + (0.183 \times cTCa) - (0.233 \times pH) + (0.0039 \times cCl) \quad (6)$$

3.3 | In vivo validation study of critically ill calves

The median and range of selected variables for 476 calves in the validated data set were similar to those presented in Table 2 for the model development data set. Passing-Bablok regression indicated a poor fit for comparing plasma $cCa^{2+}_{\text{calculated}}$ using Equations 4-6 and the measured plasma cCa^{2+} , based on the systematic differences (intercept >0) and proportional differences (slope < 1) for all 3 equations. The regression equations were:

$$cCa^{2+}_{\text{calculated}} = 0.60 + (0.50 \times cCa^{2+}) \quad (7)$$

$$cCa^{2+}_{\text{calculated}} = 0.40 + (0.67 \times cCa^{2+}) \quad (8)$$

$$cCa^{2+}_{\text{calculated}} = 0.32 + (0.73 \times cCa^{2+}) \quad (9)$$

Bland-Altman plots indicated that the 95% CIs for plasma $cCa^{2+}_{\text{calculated}}$ were -13 to +14% (Equation 7); -11 to +13% (Equation 8); and -10 to +11% (Equation 9).

The best predictive performance was obtained using all 5 variables in the final forward stepwise regression equation (Table 3), but

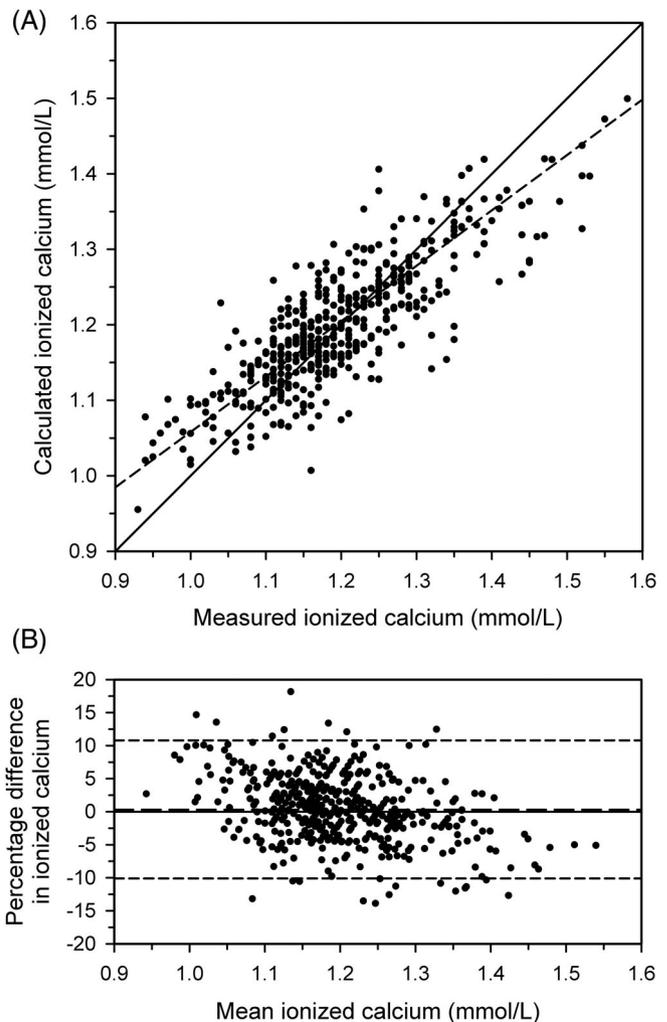


FIGURE 3 A, Scatterplot of the relationship between plasma $cCa^{2+}_{\text{calculated}}$ using the 5-factor stepwise regression equation (Table 3) and plasma cCa^{2+} measured by a direct ion-selective electrode method for 476 blood samples from critically ill calves with jugular venous blood pH > 6.90. The solid diagonal line is the line of identity, and the dashed line is the line of best fit from Passing-Bablok regression ($cCa^{2+}_{\text{calculated}} = 0.27 + 0.78 \times cCa^{2+}$). B, Bland-Altman plot of the difference between plasma $cCa^{2+}_{\text{calculated}}$ and plasma cCa^{2+} (expressed as a percentage of their mean value) against the mean value. The horizontal long dashed line is the mean bias (0.0%) and the horizontal short dashed lines reflect the 95% limits of agreement (-10.1% to +10.8%), which is equivalent to the range of differences that contains 95% of future measurements

systematic differences (intercept >0) and proportional differences (slope < 1) remained for the Passing-Bablok equation (Figure 3): $cCa^{2+}_{\text{calculated}} = 0.27 + 0.78 \times cCa^{2+}$. The associated Bland-Altman plot indicated that the 95% CI for plasma $cCa^{2+}_{\text{calculated}}$ from all 5 predictors was -10 to +10%.

4 | DISCUSSION

The experimentally determined mean value for the slope ($S = -0.227 = \Delta \log_{10} cCa^{2+} / \Delta pH$) obtained during in vitro CO_2 tonometry

of calf plasma approximated that used in many commercial blood gas, pH, and electrolyte analyzers. The value also approximated that determined previously for human albumin (-0.23)³⁶ and human serum (-0.23 ± 0.05 ; -0.24 ± 0.04).^{18,34} The following equations therefore can be used to correct $\log_{10}cCa^{2+}_m$ or cCa^{2+}_m in bovine plasma to a pH of 7.40, such that:

$$\log_{10}cCa^{2+}_{7.40} = \log_{10}cCa^{2+}_m - 0.23 \times (7.40 - pH_m) \quad (10)$$

$$cCa^{2+}_{7.40} = cCa^{2+}_m \times 10^{-0.23 \times (7.40 - pH_m)} \quad (11)$$

Both equations are valid for calf plasma with cAlbumin (≈ 31 g/L), cTotal protein (≈ 56 g/L), cCl^- (≈ 101 mmol/L), and ionic strength within the reference range. An equivalent equation for correcting cCa^{2+} in bovine plasma for change in pH from 7.40 is developed from Equation (3):

$$cCa^{2+}_{7.40} = cCa^{2+}_m \times (1 - 0.52 \times [7.40 - pH_m]) \quad (12)$$

The calculated value for B ($2.303 \times S = -0.52$) obtained during in vitro CO_2 tonometry of calf plasma approximated that determined previously for human albumin (-0.48)²³ and plasma solutions (-0.53).⁷ These corrective equations only should be used to correct for the loss of CO_2 from calf plasma or blood samples that were not anaerobically collected or stored.

We confirmed our first hypothesis that the value for S in calf plasma was similar to that of human plasma, but the value for S in undiluted calf plasma indexed to cTotal protein (-0.0040 per gram per liter of total protein; 95% CI, -0.0042 to -0.0038) was numerically higher than that for human plasma (-0.0034 per gram per liter of total protein at cTotal protein = 70.2 g/L).¹⁶ This finding suggests different calcium binding behavior for bovine and human albumin and that species-specific values for S and B are required. Calcium is bound to negative charges on plasma proteins, particularly albumin, by a pH-dependent low affinity electrostatic (salt-type) interaction.³⁶ The most likely sites for calcium binding on bovine albumin are imidazole groups that are adjacent to carboxyl groups.^{1,37-39} Alkalinization changes the net positive charge of imidazole groups and consequently influences the salt-type binding of calcium to carboxyl groups.^{39,40} The pH-induced change in net imidazole charge is believed to lead to conformational changes in the albumin molecule that may cover or uncover 2 adjacent negatively charged carboxyl sites available for salt-type binding of calcium.^{39,41}

Our findings confirmed our second hypothesis that the in vivo value for S in critically ill calves (-0.14) was lower than the experimentally determined in vitro value (-0.23) for calf plasma (coefficient for the $\Delta\log_{10}cCa^{2+}$ -pH relationship in Table 2). The lower value for S in vivo compared to in vitro has been attributed to equilibration of calcium throughout the extracellular space with an effective cAlbumin that is less than that of the intravascular space.¹⁶ However, the lower in vivo value for S may be due, in part, to differences resulting from analyzing plasma and blood. The value for S is -0.23 in human plasma

and -0.19 in whole blood,¹⁵ and our in vitro estimate was derived from plasma samples, whereas our in vivo estimate was derived from blood samples. The lower value for S in whole blood is unlikely to result from calcium binding to hemoglobin because calcium does not bind to human hemoglobin³⁴ and presumably does not bind to bovine hemoglobin.

Lower values for cAlbumin and cTotal Protein result in lower values for the slope value ($\Delta\log_{10}cCa^{2+}/\Delta pH$) of human serum.^{9,16,23} We obtained a similar finding in our study; halving of the plasma concentration of albumin and total protein during equivolume dilution decreased the mean slope value for $\Delta\log_{10}cCa^{2+}/\Delta pH$ from -0.227 to -0.146 . This finding confirmed our third hypothesis that calf plasma with decreased cAlbumin has lower in vitro values for S than does calf plasma with plasma cAlbumin within the reference range. This finding suggests that correction of the slope value for changes in cAlbumin may be required for calf plasma and is contrary to earlier suggestions that correction of cTCa for changes in cAlbumin may not be necessary for bovine plasma,²² particularly in clinical applications. However, the binding of calcium to bovine albumin is a complex process characterized by multiple binding sites that have variable affinity and binding capacity.⁴² Consequently, cCa^{2+} depends in a nonlinear manner on both cTCa and cAlbumin, particularly in hypoalbuminemia.⁴² Moreover, univariate regression of clinicopathologic data in our study indicated that although cAlbumin had a significant effect on cCa^{2+} , the effect was weak relative to that of cTCa, pH, and cCl.

Mixed models analysis of the in vitro CO_2 tonometry of equivolume-diluted plasma samples with different cCl confirmed our fifth hypothesis that increased plasma cCl directly increased plasma cCa^{2+} in cattle, similar to previous observations in humans, dogs, and rats.^{8,26-28} The increase in plasma cCa^{2+} occurred because some of the additional chloride binds to albumin and displaces bound calcium.^{24,25} An important finding of our study was that the calculated value for $cCa^{2+}_{7.40}$ in calf plasma increased by 0.0053 mmol/L (in vivo univariate analysis) for every 1 mmol/L increase in cCl. The in vivo multivariate coefficient of 0.0028 was less than the in vivo univariate coefficient of 0.0053 because an increased plasma cCl with cNa held constant decreases the plasma strong ion difference and therefore decreases plasma pH.^{25,43} Our finding that hyperchloremia directly increased plasma cCa^{2+} , independent to its effect on pH as demonstrated by in vitro equivolume dilution and multivariate analysis of in vivo data, was consistent with the results of a study in cows in which IV administration of $CaCl_2$ resulted in higher ionized plasma Ca concentrations than when the same amount of calcium (20 mg/kg body weight) was administered as a calcium gluconate formulation.⁴⁴ An increase in cCa^{2+} has been reported in studies where acidogenic rations were fed to sheep,⁴⁵ cattle,⁴⁶ and horses.⁴⁷ In these studies, a diet-induced increase in serum cCl was associated with an increase in cCa^{2+} . These observations were consistent with chloride-induced displacement of bound calcium from albumin.

A higher value for S (-0.36) has been obtained when pH is changed in vitro by non-respiratory causes, such as the addition of HCl.⁷ Interestingly, the mean slope $\Delta\log_{10}cCa^{2+}/\Delta pH$ of the linear regression equation relating $\log_{10}cCa^{2+}$ to pH differs depending on the

method used to change pH. For example, calculated *in vitro* *S* values for human serum are -0.24 or -0.21 for CO_2 tonometry, -0.22 for L -lactate titration, -0.36 for HCl titration,^{7,16,26} -0.23 for NaOH titration of human albumin solutions,³⁶ and -0.37 for $\text{PO NH}_4\text{Cl}$ ingestion.⁴⁸ Of interest is the marked difference in the measured *S* value for *in vitro* titration using HCl (-0.36)¹⁶ or NaOH (-0.23).³⁶ The higher slope value obtained during *in vitro* HCl titration or $\text{PO NH}_4\text{Cl}$ ingestion was consistent with the findings of our study that the additional chloride displaces bound calcium from albumin and increases Ca^{2+} . Addition of HCl simultaneously decreases pH and increases plasma cCl which displaces calcium from salt-type binding sites on albumin, whereas preferential absorption of chloride from the gastrointestinal tract from dissociated NH_4Cl increases plasma cCl which displaces calcium from salt-type binding sites on albumin and decreases plasma strong ion difference thus decreasing plasma pH. In both cases, the resultant hyperchloremia produces a higher apparent slope value for the $\log_{10}\text{cCa}^{2+}$ -pH relationship than that produced by changes in pH alone.

The slight decrease in the slope value during lactic acid titration, compared to CO_2 tonometry,¹⁶ may be caused by the formation of calcium-lactate complexes. Calcium complexes with L -lactate and uremic anions (eg, sulfate, phosphate) at physiologic pH and with bicarbonate and carbonate at nonphysiologic pH, primarily when $\text{pH} > 7.9$.^{10-12,17,49} We attribute our finding that plasma L -lactate concentration was negatively associated with plasma cCa^{2+} to the *in vivo* formation of cationic calcium- L -lactate complexes, such as Ca-L-lactate^+ .⁵⁰ This potentially clinically relevant association has been minimally investigated. A preliminary report in 9 critically ill humans⁵¹ and *in vitro* studies involving human blood⁵⁰ and aqueous solutions⁵² document clinically relevant binding of L -lactate to calcium. A study in exercising humans calculated that the amount of ionized calcium bound to L -lactate increased from 0.016 mmol/L (1.3% of $\text{cCa}^{2+} = 1.22$ mmol/L) at $\text{cLactate} = 1$ mmol/L to 0.100 mmol/L (7.5% of $\text{cCa}^{2+} = 1.34$ mmol/L) at $\text{cLactate} = 9.2$ mmol/L.⁵³ Similarly, our finding that plasma cMg was negatively associated with plasma cCa^{2+} most likely reflected the *in vivo* formation of poorly soluble anionic uremic salts such as calcium sulfate⁴⁹ because cMg, and presumably sulfate, is increased in azotemic calves.⁵⁴

The cCa^{2+} reflects the activity of calcium ions in solution, and the measured value consequently is dependent on the temperature and ionic strength of the solution. The temperature effect is accounted for during analysis because cCa^{2+} most commonly is measured at 37°C by blood pH, gas, and electrolyte analyzers. The major determinant of ionic strength in plasma is the plasma Na concentration (cNa), and the ionized cCa^{2+} has been shown experimentally to vary directly with cNa because increased ionic strength decreases the binding of calcium to albumin.⁵⁵ A large range of values for plasma cNa (110-188 mmol/L) was observed in the critically ill calves in our study, and univariate analysis indicated that plasma cNa^+ was positively associated with cCa^{2+} .

Our study design utilized a focused *in vitro* study followed by an *in vivo* study of a large number of critically ill calves from multiple farms with a randomly assigned hold-out population sample for model confirmation and as such had many strengths. Additional *in vitro* and *in vivo* studies in adult dairy cows are indicated to confirm the

absence of age effects on cCa^{2+} and to explore the potential impact of hyperketonemia and increased plasma non-esterified fatty acid concentration on plasma cCa^{2+} . Increased plasma non-esterified fatty acid concentration in humans increases the binding of calcium to albumin and decreases serum cCa^{2+} .⁵⁶

In conclusion, our findings indicate that cCa^{2+} in calf plasma primarily is dependent on cTCa, blood pH, and cCl. As such, calculation of cCa^{2+} from measured cTCa requires adjustment for at least pH and cCl when 1 or both are outside the reference range and possibly adjustment for cMg as a proxy for the presence of uremic anions that can complex ionized calcium and L -lactate. However, it must be emphasized that the clinical relevance of *in vivo* changes in pH and plasma cCl on *in vivo* plasma cCa^{2+} is small, in that Equation 6 predicts that a 0.2 unit decrease in pH will increase cCa^{2+} by only 0.047 mmol/L (3.9% of a reference mean value of 1.2 mmol/L), whereas a 10 mmol/L increase in plasma cCl increases cCa^{2+} by only 0.039 mmol/L (3.3% of a reference mean value of 1.2 mmol/L). Nevertheless, the *in vivo* effect of a 10 mmol/L increase in cCl approximates that induced by a 0.2 unit decrease in pH, suggesting that it is inappropriate to focus corrective equations for cCa^{2+} only on changes in pH unless the sample was not collected and stored in an anaerobic manner. Although calculation of cCa^{2+} from 5 predictors produced a value with a 95% CI of 10%, our findings provide a potential explanation for why "corrective" equations that include pH and plasma cAlbumin or cTotal protein but exclude cCl are poorly predictive of cCa^{2+} in human plasma,⁵⁷ canine plasma,⁵⁸ and bovine serum.⁵⁹ Our findings also were consistent with a recent study in dogs that indicated serum cCl was an important predictor of whole-blood cCa^{2+} .⁶⁰ We anticipate that the results of our study will lead to additional studies in humans and domestic animals that characterize the combined effects of pH and cCl on plasma cCa^{2+} .

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed as standard blood samples used.

In vivo study—data extracted from part of the following paper (which did not report ionized calcium concentration). Trefz FM, Feist M, Lorenz I. Hypoglycaemia in hospitalised neonatal calves: Prevalence, associated conditions and impact on prognosis. *Vet J*. 2016;217:103-108.

In vitro study—data extracted from the following study—ionized calcium concentration not reported)—Constable PD, Stämpfli HR, Navetat H, Berchtold J, Schelcher F. Use of a quantitative strong ion

approach to determine the mechanism for acid-base abnormalities in sick calves with or without diarrhea. *Journal of Veterinary Internal Medicine*. 2005;19(4):581-589.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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