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Prediction of ionized calcium concentration based on total calcium and protein levels in cattle and sheep

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Abstract

Despite being important, there are no equations for prediction of ionized calcium (iCa) in sheep and cattle. The objectives of this study were i) to create equations for the calculation of serum iCa concentration based on the serum concentrations of total calcium (tCa), albumin (Alb) and total proteins (TP) and ii) to investigate whether predicted serum iCa values are beneficial in clinical practice. Serum samples from 30 sheep and 30 dairy cattle were used. Serum tCa was determined colorimetrically, while serum iCa was determined with an ion selective electrode method. Serum Alb and TP concentration were determined using bromocresol green and biuret methods, respectively. Ionized calcium was also calculated based on serum tCa, using regression analysis, and with two equations based on Alb and TP concentration. Bland–Altman plots were plotted to evaluate the agreement between measured and predicted iCa; Passing and Bablok (P - B) regression analysis was used to assess their agreement. The initial equations were corrected using the P - B generated equation and Bland – Altman plots were run to evaluate the level of agreement between measured and predicted iCa using the final equations. Six equations were finally created for cattle and 6 for sheep. The total bias exceeded 10.00% in all of them indicating that they are clinically unacceptable for iCa prediction especially when the predicted result is very close to the cut-off point of < 1.00 mmol L^{-1}. So, it could be suggested that, when necessary, iCa concentration should be directly determined.

Introduction

Calcium is a divalent cation in the extracellular compartment contributing to many cell functions. Intracellular ionic Ca is found in very small concentrations due to the expression of membrane Ca channels and the protein bounding,[1] while calcium blood concentrations are regulated in a narrow range from the protein,[2] mainly albumin (Alb), bound fraction, total calcium is comprised of a calcium complex with various anions, and the free ionized fraction. The free ionized fraction of the cation is considered as the physiologically active, hormonally regulated form, to which the body responds.[2,3]

Hypocalcemia is a relatively frequent pathological condition in dairy cows, which occurs after calving and commonly known as milk fever. Milk fever seems to be a delayed adaptation of the metabolism to the increased and prolonged demand in calcium at parturition, rather than a nutritional or degenerative disease.[4] A similar metabolic periparturient condition also known as parturient paresis or "lambing sickness" is reported in sheep. It is observed during an interval ranging from several weeks before and until the first 2 weeks after parturition and is presented in an acute or subacute form.[5] Low blood serum total calcium (tCa) concentration confirms the diagnosis of both clinical conditions.

In clinical practice, tCa concentration is determined by employing colorimetric assays. Atomic absorption spectrophotometry, however, has been used in many studies, instead of colorimetric spectrophotometry for total calcium measurement when concentrations below the sensitivity of colorimetric assays were expected.[2,6]

Due to the large percentage of protein-bound total calcium, hypoalbuminemia can decrease tCa concentration.

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In these cases, free ionized calcium (iCa) concentration will be unaffected by the decreased Alb alone. Therefore, calcium concentration should be interpreted in combination with the protein concentration.[7]

The determination of iCa can be easily performed using ion selective electrode electrolyte or blood gas analyzers. However, these devices are not always available in small, low cost farm animal veterinary practices. To predict ionized calcium different equations, using several parameters such as protein concentration, blood pH, potassium (K), sodium (Na), have been created and evaluated in dogs,[8,9] cats,[10] and horses with conflicting results.[11] Moreover, among these parameters, protein concentration can be determined by the same basic biochemistry equipment as tCa. So, it would be of value if iCa could be estimated using the blood serum concentrations of other parameters of low cost and easily determined.

The objectives of the present study were i) to create equations for the calculation of serum iCa concentration based on the serum concentrations of tCa, Alb and total proteins (TP) and ii) to investigate whether predicted serum iCa values are acceptable for use in clinical practice.

Materials and Methods

Blood samples that were obtained from 30 Holstein cows in early lactation and 30 Chios sheep in dry period for the assessment of the risk for hypocalcemia at herd level during 2019 in the Clinic of Farm Animals of School of Veterinary Sciences of the Aristotle University of Thessaloniki were used in the study. The blood samples were anaerobically collected directly into vacuum glass tubes without anticoagulant (BD, Franklin Lakes, USA), by tail venipuncture in cattle and jugular venipuncture in sheep with an 18.00 G needle. All tubes were 100% filled with blood and the tube was withdrawn from the holder before needle removal. The serum was separated immediately after clotting by low speed centrifugation at 1,600 *g* for 10 min. Then serum was transferred into plastic vials (Eppendorf AG, Hamburg, Germany), fully filled and kept refrigerated (2.00 - 4.00 ˚C) until analysis which was performed within 24 hr. All samples used did not have any visible abnormality (hemolysis, clots). No ethical approval was obtained as this study did not involve any laboratory animals and concerned only non-invasive procedures (blood sampling, only once from each animal).

Serum iCa concentration was measured first using an ion selective electrode method in an automatic analyzer (9180 Electrolyte Analyzer; Roche Diagnostics, Indianapolis, USA). Subsequently, serum tCa concentration was determined using two methods: flame atomic absorption spectrophotometry using a Perkin-Elmer AAnalyst 300 flame atomic absorption spectrophotometer (Perkin-Elmer Corp. Norwalk, USA) according to the instructions of manufacturer and a colorimetric method in an automatic biochemical analyzer (Vitalab Flexor E; Vital Scientific NV, Dieren, Netherlands) using a commercial diagnostic kit for serum calcium (ThermoFisher Scientific Inc., Waltham, USA). Serum Alb and TP concentration were determined using bromocresol green and biuret methods respectively, in an automatic biochemical analyzer using commercial diagnostic kits (ThermoFisher Scientific Inc.).

Linear regression analysis (SPSS Software, version 25.0; IBM Corp., Armonk, USA) without using a constant in the model was run to determine the association factor (b) between serum iCa and tCa concentration in both cattle and sheep samples. Consequently, using the estimated conversion factor, serum iCa (mmol L^{-1}) was calculated based on tCa concentration determined with flame atomic absorption spectrophotometry and with the colorimetric method as follows:

$$iCa_{1zy} = b \times tCa_y \text{ (Eq. 1)}$$

Serum iCa (mmol L^{-1}) concentration was also calculated for both species using the following equations based on the serum concentration of tCa (mg dL^{-1}), Alb (g dL^{-1}) and TP (g dL^{-1}): [12,13]

$$iCa_{2zy} = 0.25 \times [0.90 + (0.55 \times tCa_y - 0.30 \times Alb)] \text{ (Eq. 2)}$$

$$iCa_{3zy} = 0.25 \times (6.00 \times tCa_y - TP / 3) / (6.00 + TP) \text{ (Eq. 3)}$$

On all above equations, the subscript z represents the animal species (cattle or sheep) and the subscript y the method of tCa determination (atomic absorption spectrophotometry or colorimetric method).

Bland - Altman plots[14] were used to evaluate the agreement between measured and calculated iCa values. Passing and Bablok (P - B) regression analysis[15,16] was also run to assess the agreement between them. In order to create new predictive equations for iCa that could have improved performance, the initial equations used were incorporated in the generated P - B equations. Bland - Altman plots were run again to evaluate the level of agreement between measured and calculated iCa using the final equations. The statistical software MedCalc Software (version 9.2; MedCalc, Ostend, Belgium) was used for these comparisons.

Results

Using the cut-off of ≤ 2.00 mmol L^{-1} for tCa, four and three cattle were classified as hypocalcemic based on the results of flame atomic absorption spectrophotometry and the colorimetric method, respectively. According to iCa results, three cows were hypocalcemic using as cut -off value < 1.00 mmol L^{-1}. The average iCa concentration for this species was 1.07 mmol L^{-1} (SD: 0.07; min - max: 0.89 - 1.20 mmol L^{-1}). In sheep, no ewe was classified as hypocalcemic using the same cut-off for tCa, whereas 25 sheep were considered as hypocalcemic based on iCa (≤ 1.00 mmol L^{-1}). The average iCa concentration for the

ewes used in the study was 0.91 mmol L^{-1} (SD: 0.10; min-max: 0.65 - 1.10 mmol L^{-1}). Serum Alb and TP concentrations were all within the reference range of our laboratory in both cattle and sheep.

Based on the results of linear regression analysis and the determination of b factors for Eq. 1, the four equations used for the calculation of iCa based on tCa alone in cattle and sheep and the associated biases according to Bland and Altman plots are presented in Table 1. The respective P - B equations generated were as below:

for cattle

$$iCa = 0.55\ (95.00\%\ CI: 0.04 – 0.75) + 0.50\ (95.00\%\ CI: 0.30 – 0.96) \times iCa_{1cat}$$

$$iCa = 0.46\ (95.00\%\ CI: – 0.21 – 0.82) + 0.58\ (95.00\%\ CI: 0.24 – 1.20) \times iCa_{1cc}$$

and for sheep:

$$iCa = – 0.24\ (95.00\%\ CI: – 1.60 – 0.28) + 1.29\ (95.00\%\ CI: 0.71 – 2.77) \times iCa_{1sat}$$

$$iCa = 0.00\ (95.00\%\ CI: – 3.18 – 0.46) + 1.00\ (95.00\%\ CI: 0.50 – 4.50) \times iCa_{1sc}$$

The final equations obtained by the incorporation of iCa values of Table 1 on these equations and the relevant biases are shown on Table 2 with the exception of the last equation for sheep which finally generates Eq. 1 SC.

The calculation of iCa with Eq. 2 is associated with average total bias in cattle -16.70% (1.96 SD: – 35.40% – 2.00%) and – 10.00% (1.96 SD: – 29.20% – 9.20%) using tCa values determined with atomic absorption spectro-photometry and colorimetric method, respectively. In sheep, the average total biases are – 41.40% (1.96 SD: – 64.30% – 18.60) and – 22.20% (1.96 SD: – 49.10% – 4.70%) for tCa values determined with atomic absorption spectrophotometry and colorimetric method, respectively.

The P - B equations between measured and calculated iCa with Eq. 2 are as follow:

for cattle

$$iCa = 0.58\ (0.10 – 0.76) + 0.40\ (0.25 – 0.75) \times iCa_{2cat}$$

$$iCa = 0.42\ (– 0.27 – 0.76) + 0.56\ (0.27 – 1.14) \times iCa_{2cc}$$

and for sheep:

$$iCa = 0.11\ (– 0.80 – 0.55) + 0.59\ (0.27 – 1.24) \times iCa_{2sat}$$

$$iCa = 0.07\ (– 1.73 – 0.53) + 0.74\ (0.36 – 2.30) \times iCa_{2sc}$$

The use of these equations after the incorporation of Eq. 2 in them generated the predictive equations and the biases presented in Table 3.

The use of Eq. 3 that takes into account the serum total protein concentration for the calculation of iCa is associated with very high average total bias or high standard deviation of difference and is not considered as acceptable for iCa estimation in either cattle [– 1.00% (1.96 SD: – 19.70% – 17.70%) and 6.00% (1.96 SD: –11.70% – 23.80%) using tCa values determined with atomic absorption spectrophotometry and colorimetric method, respectively] or sheep [– 29.40% (1.96 SD: –57.90% – 0.80%) and – 9.70% (1.96 SD: –34.20% – 14.70%) using tCa values determined with atomic absorption spectro-photometry and colorimetric method, respectively]. Taking into consideration the generated P - B equations;

for cattle:

$$iCa = 0.58\ (0.17 – 0.77) + 0.45\ (0.29 – 0.83) \times iCa_{3cat}$$

$$iCa = 0.17\ (-0.41 – 0.64) + 0.91\ (0.44 – 1.50) \times iCa_{3cc}$$

and for sheep:

$$iCa = 0.31\ (– 0.30 – 0.60) + 0.51\ (0.26 – 1.00) \times iCa_{3sat}$$

$$iCa = -0.54\ (– 8.17 – 0.24) + 1.43\ (0.67 – 9.00) \times iCa_{3sc}$$

The equations shown in Table 4 were created.

Using the cut-off point of iCa < 1.00 mmol L^{-1}, the number of correctly and falsely predicted as hypocalcemic cases using the final equations presented in tables 1 to 4 for cattle and sheep as well as the respective sensitivity and specificity are presented in Table 5.

Table 1. Regression equations (Eq. 1 CA, Eq. 1 CC, Eq. 1 SA, Eq. 1 SC) generated using linear regression model for the calculation of ionized calcium (iCa) using serum total calcium (tCa) with coefficient of determination (R^2) and average total bias (± 1.96 SD), determined with Bland - Altman plots, between measured iCa values and those calculated with each equation in cattle and sheep.

Species	Equation	Regression equations	R^2	Average bias (± 1.96 SD) %
Cattle	**1CA**	$iCa_{1cat} = 0.45 \times tCa_{at}$	0.99	0.70 (– 16.70 – 18.10)
	1CC	$iCa_{1cc} = 0.49 \times tCa_{c}$	0.99	1.00 (– 19.40 – 21.40)
Sheep	**1SA**	$iCa_{1sat} = 0.35 \times tCa_{at}$	0.99	0.20 (– 22.80 – 23.20)
	1SC	$iCa_{1sc} = 0.42 \times tCa_{c}$	0.98	1.20 (– 26.80 – 29.30)

iCa_{1cat} = iCa values calculated for cattle using tCa values determined with atomic absorption spectrophotometry (tCa_{at}); iCa_{1sat} = iCa values calculated for sheep using tCa values determined with atomic absorption spectrophotometry (tCa_{at}); iCa_{1cc} = iCa values calculated for cattle using tCa values determined with the colorimetric method (tCa_c); iCa_{1sc} = iCa values calculated for sheep using tCa values determined with the colorimetric method (tCac).

Table 2. Final predictive equations for ionized calcium (iCa) estimation in cattle (Eq. 4, Eq. 5) and sheep (Eq. 6) using serum total calcium values determined either with atomic absorption spectrophotometry (tCa_{at}) or the colorimetric method (tCa_c) and respective average total bias (± 1.96 SD), determined with Bland-Altman plots, between measured iCa values and those calculated with each equation.

Species	Equation	Predictive equations	Average total bias (± 1.96 SD) %
Cattle	**4**	$iCa = 0.55 + 0.23 \times tCa_{at}$	– 1.00 (– 13.10 – 11.10)
	5	$iCa = 0.46 + 0.28 \times tCa_{c}$	0.80 (– 16.00 – 14.40)
Sheep	**6**	$iCa = – 0.24 + 0.45 \times tCa_{at}$	–1.20 (– 27.00 – 24.60)

Table 3. Final predictive equations for ionized calcium (iCa) estimation in cattle (Eq. 7, Eq. 8) and sheep (Eq. 9, Eq. 10) using serum albumin concentration (Alb) and serum total calcium values determined either with atomic absorption spectrophotometry (tCa_{at}) or the colorimetric method (tCa_c) and respective average total bias (± 1.96 SD), determined with Bland - Altman plots, between measured iCa values and those calculated with each equation.

Species	Equation	Predictive equations	Average total bias (± 1.96 SD) %
Cattle	7	$iCa = 0.67 + 0.06 \times tCa_{at} - 0.03 \times Alb$	–1.90 (– 14.20 – 10.40)
	8	$iCa = 0.55 + 0.08 \times tCa_c - 0.04 \times Alb$	– 1.80 (– 16.80 – 13.20)
Sheep	9	$iCa = 0.24 + 0.08 \times tCa_{at} - 0.04 \times Alb$	– 0.60 (– 23.50 – 22.20)
	10	$iCa = 0.24 + 0.14 \times tCa_c - 0.08 \times Alb$	1.10 (– 24.90 – 27.20)

Table 4. Final predictive equations for ionized calcium (iCa) estimation in cattle (Eq. 11, Eq. 12) and sheep (Eq. 13, Eq. 14) using serum total proteins (TP) concentration and serum total calcium values determined either with atomic absorption spectrophotometry (tCa_{at}) or the colorimetric method (tCa_c) and respective average total bias (± 1.96 SD), determined with Bland - Altman plots, between measured iCa values and those calculated with each equation.

Species	Equation	Predictive equations	Average total bias (± 1.96 SD) %
Cattle	11	$iCa = 0.58 + 0.11 \times (6.00 \times tCa_{at} - TP / 3) / (6.00 + TP)$	– 0.10 (– 12.40 – 12.30)
	12	$iCa = 0.17 + 0.23 \times (6.00 \times tCa_c - TP / 3) / (6.00 + TP)$	–1.60 (– 17.90 – 14.60)
Sheep	13	$iCa = 0.31 + 0.13 \times (6.00 \times tCa_{at} - TP / 3) / (6.00 + TP)$	– 2.30 (– 25.30 – 20.70)
	14	$iCa = -0.54 + 0.36 \times (6.00 \times tCa_{at} - TP / 3) / (6.00 + TP)$	2.60 (– 29.60 – 34.80)

Table 5. Number of cases that were classified as truly or falsely hypocalcemic (true or false positive, respectively) or normal (true or false negative) according to the equations presented in tables 1 to 4 in comparison with the true hypocalcemic cases in cattle and sheep based on measured serum ionized calcium using the cut-off of ≤ 1 mmol L^{-1}.

Species	Equation	True positive	False positive	True negative	False negative	Sensitivity (%)	Specificity (%)
Cattle	1 CA	1.00	4.00	23.00	2.00	33.33	85.20
	1 CC	2.00	7.00	20.00	1.00	66.67	74.07
	4	1.00	2.00	25.00	2.00	33.33	92.59
	5	0.00	1.00	26.00	3.00	0.00	96.30
	7	0.00	1.00	26.00	3.00	0.00	96.30
	8	0.00	1.00	26.00	3.00	0.00	96.30
	11	0.00	3.00	24.00	3.00	0.00	88.89
	12	1.00	2.00	25.00	2.00	33.33	92.59
Sheep	1 SA	23.00	4.00	1.00	2.00	92.00	20.00
	1 SC	23.00	5.00	0.00	2.00	92.00	0.00
	6	21.00	3.00	2.00	4.00	84.00	40.00
	9	24.00	3.00	2.00	1.00	96.00	40.00
	10	23.00	5.00	0.00	2.00	92.00	0.00
	13	22.00	2.00	3.00	3.00	88.00	60.00
	14	20.00	5.00	0.00	5.00	80.00	0.00

Discussion

The objective of the present study was to generate and evaluate equations for the calculation of serum iCa concentration in cattle and sheep. We used serum samples of the first days of lactation in cattle and during the dry period in sheep because these are the productive stages with the highest risk for hypocalcemia in these species and consequently, the most valuable time for serum iCa determination.[17,18] In order to avoid the effects of anti-coagulants, air content, and carbon dioxide in iCa measurements,[19-22] we used sera instead of plasma and we paid special attention to ensure, as much as possible, the anaerobic handling of samples by filling the tubes at their total capacity. The calculations were made using tCa values determined with both atomic absorption spectrophotometry and the colorimetric method because the first one is the reference method for calcium determination and the second one the most commonly used due to its lower cost. We only incorporated in the equations only the factors that directly affect iCa concentrations such as tCa, Alb and TP concentrations. These parameters are very commonly determined for the health evaluation of ruminants, while the use of a model with additional parameters would increase the cost and would make such an evaluation not cost-effective compared to the direct measurement of iCa.

In cattle samples, four were classified as hypocalcemic according to flame atomic absorption spectrophotometry and 3 based on those of the colorimetric method. This discrepancy is due to the fact that tCa values in one animal were very close to the cut-off point of 2.00 mmol L^{-1}. The iCa values measured in both cattle and sheep samples were very close to 1.00 mmol L^{-1} which is the cut-off point used for hypocalcemia. This was rather expected taking into account the physiological stage of the animals included in the study. The difference on the number of

sheep classified as hypocalcemic according to iCa and tCa indicates that factors other than tCa also affect the iCa concentration and denotes the necessity of iCa evaluation in animals at risk for hypocalcemia.

In order to assess the direct association between tCa and iCa in both species, the regression equations of Table 1 were created without including constant in the model. The fact that the coefficient of determination is very close to 1 in all equations suggests that the variability of the ionized calcium is very well explained by the variability of tCa. These equations denote that iCa represents lower percentage of serum tCa in sheep compared to cattle. This finding is in agreement with former observations since the recorded rates are very close to 50.00% reported for cattle[23-25] and similar to approximately 41.00% referred for sheep.[26]

From analytical point of view, the total allowable error for iCa determination is 2.00%.[27] However, an error of less than 10.00% could be acceptable in clinical practice. Despite the fact that the average bias in the equations of Table 1 is less than 2.00%, the range is higher than 10.00%. The use of equations 2 and 3 are also associated with very high average and total bias. In addition, all P - B equations generated, with the exception of that based on tCa measured with the colorimetric method for sheep, revealed constant and proportional errors suggesting that predicted and measured iCa cannot be used interchangeably. Thus, all these equations are not regarded as acceptable for the correct prediction of iCa in either cattle or sheep. Similar results were obtained in a former study in dogs.[28]

In both species, it was observed that taking into account either serum Alb or serum TP levels on iCa prediction does not improve the bias of calculation over the equations using tCa alone. In addition, in sheep, due to the lower percentage of iCa as fraction of tCa, it was hypothesized that equations involving Alb or TP might be more sufficient for the prediction of iCa values but this hypothesis was not confirmed. These observations probably indicate that the variability in calcium bound to non-protein anions could also affect the accuracy of the predictive equations. They could also be attributed to the fact that all animals of this study had normal Alb and TP values. It is uncertain how this calculation could have been affected if the animals had been hypoproteinemic or hyperproteinemic.

In an attempt to minimize the observed errors and to improve the prediction of iCa, the generated P - B regressions were incorporated in the initial equations for retracting the observed biases. It is well known that this practice increases the risk of overfitting and leads to overoptimistic performance. However, it was followed to explore the possibility and the degree of performance improvement. The obtained results indicate that the use of the generated equations did improve the total observed bias in all equations. However, they are not regarded as clinically acceptable especially when the predicted iCa values are close to the cut-off point of 1.00 mmol L^{-1} as observed on the study population. This is further confirmed by the remarkable number of cases that are misclassified based on the predicted iCa values using the final equations as it is shown in Table 5.

The results of the present study indicate that the prediction of iCa using the equations of Table 1 and the equations 2 and 3 is associated with high total bias and is not acceptable to be used in clinical practice . In addition, the generated equations of Tables 2-4, despite the lower total error, are also not considered as clinically acceptable for iCa prediction especially when the predicted result is very close to the cut-off point of < 1.00 mmol L^{-1}. Therefore, it could be suggested that, when necessary, iCa concentration should be directly determined.

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Conflict of interest

The authors declare no conflict of interests.

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