



Changes in ionized calcium concentration in the blood of dairy cows with peracute coliform mastitis

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ABSTRACT. We determined the clinical signs and blood ionized calcium (iCa) levels in dairy cows with peracute coliform mastitis (PCM). The clinical scores at the onset of the disease (day 0) and on day 2 and subsequent days were significantly ($P < 0.01$) higher than those of healthy cows. We found a positive correlation ($r = 0.894$, $P < 0.01$) between iCa and total calcium (TCa) concentrations in the blood of healthy cows; however there was no correlation from day 0 to day 3 in the blood of PCM cows. Multiple regression analysis revealed that the concentration of iCa was correlated with rectal temperature, hematocrit value, platelet count, and albumin level of PCM cows at the onset of disease ($r = -0.804$, $r = 0.6576$, $r = 0.6182$, $r = 0.284$, $P < 0.01$, respectively). There was no correlation between the TCa concentration and these parameters for PCM cows at day 0. Low blood iCa concentration at day 0 for PCM cows was related to symptoms of septic shock involving hypothermia, activation of the blood coagulation system, and dehydration.

KEY WORDS: dairy cow, ionized calcium, peracute coliform mastitis

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Mastitis is a major cause of economic loss in dairy farms worldwide [1, 23]. Although staphylococci and environmental streptococci cause mastitis, coliform bacteria typically cause more severe cases [24]. Dairy cows with acute coliform mastitis caused by *Escherichia coli* (*E. coli*) exhibit a wide range of systemic clinical features. These symptoms can include rumen stasis, depression, pyrexia, anorexia, circulatory disturbance, marked decrease in milk production, and death [23]. Peracute coliform mastitis (PCM) manifests as serious symptoms in dairy cows and causes disseminated intravascular coagulation syndrome from bacteremia and sepsis [4, 14, 26].

Severe acute coliform mastitis displays significantly lower hypocalcemia than that of mild cases [29]. The bones represent <1% of total body calcium (10 g in an adult). Serum calcium ranges from 8.8 to 10.4 mg/dl (2.2 to 2.6 mM) in healthy subjects [18]. Calcium in the blood circulates in three forms, consisting of 54% ionized calcium (iCa), 33% protein-bound calcium, and 13% calcium bound to phosphoric acid, bicarbonate, and organic acids [20, 21]. A hormonal pathway maintains homeostasis of serum calcium levels through a rapid negative feedback mechanism that senses the serum iCa concentrations and parathyroid hormone (PTH) secretion from the parathyroid [18]. Calcium receptors in the parathyroid cells inactivate when serum calcium levels fall, triggering an increase in PTH secretion. This increase in PTH balances serum calcium by activating the parathyroid receptors in the bones and kidneys, increasing overall calcium resorption. In the kidney, increased PTH secretion enhances calcium-restorative effects by increasing secretion of 1,25-dihydroxyvitamin D (1.25D), the vitamin D receptor in the gut, and increases active calcium absorption by the bone [18].

Hypocalcemia is commonly found in human patients with severe sepsis and burns. In these cases, the proinflammatory cytokines IL-1 β and IL-6 up-regulate calcium-sensing receptor (*CASR*) expression in the parathyroid and kidneys through a defined response of elements in the *CASR* gene promoters. This results in decreased serum PTH and 1,25-D and calcium levels [9]. Zhang *et al.* reported that cows with latent hypocalcemia also have low Ca²⁺ in neutrophils and reduced neutrophil migration, adhesion, and phagocytosis [25]. We have observed hypocalcemia even at our initial visit to PCM cows. Leno *et al.* reported the relationship

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between blood total calcium (TCa) and iCa of postpartum cows [15]. However, we are unaware of a study related to clinical signs and iCa and TCa concentrations in the blood of PCM cows.

The purpose of this study was to measure iCa and TCa concentrations in the blood of PCM cows and to determine the relationships among the clinical signs, blood parameter values, and the iCa and TCa concentrations in blood.

MATERIALS AND METHODS

Cows

For this study, 11 Holstein dairy cows were vaccinated with a mastitis vaccine (Startvac[®] HIPRA UK, Ltd., Girona, Spain) and with naturally occurring PCM from three local dairy farms in the Nanyo District, Ehime. The vaccine was injected intramuscularly 45 days prior to, 10 days prior to, and 52 days after parturition [7]. The numbers of treated cows were as follows: two cows within 7 weeks after parturition, and three cows from 7 to 15 weeks, three cows from 16 to 28 weeks, and three cows from 29 to 42 weeks after parturition. During the clinical course of mastitis, we diagnosed PCM cows based on the clinical findings of fever or hypothermia ($\geq 39.5^{\circ}\text{C}$ or $< 38.5^{\circ}\text{C}$, respectively), anorexia, induration, and swelling of the udder, and identified coliform bacteria by bacteriological culture. We matched treated cows with eight healthy Holstein dairy cows. The PCM cows consisted of two cows from 3 years old, which were within 3 weeks after parturition, and three cows from 3 to 18 weeks and three cows from 19 to 38 weeks after parturition. Cows were properly handled according to the “Guidelines for Proper Implementation of Animal Experiments” established by the Science Council of Japan.

Scoring clinical signs

Scoring of clinical symptoms was performed from day 0 to day 3. The clinical signs observed included anorexia, cold extremities, loss of pinna reflex, diarrhea, ocular depression, dysstasia or anastasia, swelling of the udder, induration, watery milk, milky discoloration, and flakes in milk. The presence of each sign was assigned one point and the total of these points was the clinical score.

Pre- and post-healing (two weeks) of the milk yield of PCM cows was recorded from farmer interviews as the dairy herd improvement test.

The PCM cows were prognostically determined 2 weeks after the end of treatment.

Treatment of PCM cows

We treated PCM cows on day 2 and day 3. Affected cows with PCM were treated with an intravenous injection of the antibiotic enrofloxacin (5 mg/kg, 10% Bitril, Bayer Pharmaceutical Co., Ltd., Osaka, Japan) with fluid therapy, including hypertonic saline solution (2–3 l, Kawasaki Mitaka Pharmaceutical Co., Ltd., Kawasaki, Japan). We administered 10 ml of aqueous dexamethasone (Aqueous dexamethasone injection A, Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan) subcutaneously as an anti-inflammatory agent. No calcium agent was given during any treatment.

Blood collection and examination

Peripheral blood was collected from the jugular vein into tubes with anticoagulant (Beneject II vacuum blood collection tubes, Terumo Co, Tokyo, Japan) on days 0, 2, and 3 of both PCM and healthy cows. We immediately measured the iCa concentration in the blood using the iCa checker (Compact Bovine Blood iCa Checker, Horiba Co., Kyoto, Japan). We used an automatic hemocytometer (Celltaca, Nihonkohden, Tokyo, Japan) to determine leukocyte counts, platelet counts (PLT), and hematocrit values (Ht). The TCa concentration, and blood urea nitrogen (BUN), total protein (TP), total cholesterol (T-cho), albumin (Alb), inorganic phosphorus (iP), and magnesium (Mg) levels in the blood were measured using an auto-analyzer (AU480, Beckman Coulter, Brea, CA, USA). We calculated the A/G in the blood as $\text{Alb}/(\text{TP}-\text{Alb})$.

Milk collection and examination

We collected milk samples from the mastitic quarters of PCM cows on the first day. Milk samples (10 μl) were plated onto trypticase soy blood agar containing 5% sheep blood (Trypticase Soy Agar with 5% Sheep Blood, Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) and McConkey agar (Nissui Plate MacConkey Agar, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and incubated aerobically for 18 hr at 37°C . Pathogens that grew on the plates were counted and coliform bacteria were identified using a commercially available kit (ID test EB-20, Nissui Pharmaceutical Co., Ltd.).

The number of somatic cells in milk samples of healthy and PCM cows was measured using a somatic cell counter (Cell Counter DCC, Delaval, Tokyo, Japan) from day 0 to day 3.

Statistical analysis

All data are expressed as the mean standard deviation (SD). A one-way ANOVA with Bonferroni's test was used for the statistical analysis of rectal temperatures and leukocyte counts. The Kruskal-Wallis test was used to statistically analyzed differences in heart rate, respiration rate, clinical score, hematocrit values, platelet counts, BUN, TP, T-cho, Alb, iP, Mg, and TCa and iCa blood concentrations, and the number of somatic cells in the milk of PCM cows and healthy cows. Levels of iCa and TCa in the blood of healthy and PCM cows were examined for correlations using a single regression line. The causal relationships between rectal temperature, hematocrit values, platelet counts, and albumin and iCa concentrations in the blood in PCM cows on

day 0 were determined by multiple regression analysis. The Mann-Whitney-test was used to determine differences in milk yield before day 0 and milk yield after disease resolution in PCM cows. All statistical analyses were performed using Excel Tokei 2015 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Statistical significance was defined as $P < 0.05$.

RESULTS

Clinical findings and milk yield in healthy and PCM cows

We recorded seven *E. coli* and four *Klebsiella pneumoniae* infections in PCM cows. Eleven PCM cows were cured and one infection resulted in death (5 days after onset of illness). Table 1 summarizes the clinical signs of healthy cows and PCM cows from day 0 until day 3. The heart rate of PCM cows at day 0 was significantly greater ($P < 0.01$) than that of healthy cows and PCM cows on day 2. The clinical score of PCM cows on day 0 was significantly higher ($P < 0.01$) compared to that of the healthy cattle and on day 2 and day 3 of PCM cows. There were no significant differences ($P = 0.2737$) in milk yield (30.9 ± 4.9 kg) after 2 weeks of the 10 cured PCM cows compared to the yield before the illness (33.1 ± 5.3 kg). The hematological examination of PCM and healthy cows are shown in Table 2. The leukocyte counts on day 0 for PCM cows were significantly ($P < 0.05$) lower than on day 3. BUN measurements on day 0 of PCM cows were significantly ($P < 0.05$) higher than those of healthy cows and day 3 PCM cows. The blood TCa concentration on day 0 for PCM cows was significantly ($P < 0.05$) lower than that of healthy cows. There was no significant difference in the blood iCa concentrations of the healthy cows and that of day 0 until day 3 for PCM cows. The number of somatic cells in the milk on day 0 to day 3 of PCM cows was significantly ($P < 0.01$) higher than that of healthy cows.

There was a positive correlation ($r = 0.894$, $P < 0.01$) between iCa and TCa concentrations in healthy cows. However, there was no correlation for PCM cows from day 0 to day 3 (Fig. 1).

Relationships between iCa concentrations, clinical signs, and hematological parameters of PCM cows

In the multiple regression analysis, a correlation was found between iCa on day 0 and hematocrit values, platelet counts, rectal temperature, and albumin levels ($r = -0.8040$, $r = 0.6576$, $r = 0.6182$, $r = 0.2840$, $P < 0.01$, respectively) (Table 3). No correlation was detected for TCa concentrations of PCM cows at day 0 and these values ($r = -0.6017$, $r = 0.6395$, $r = 0.5190$, $r = 0.1038$, $P = 0.2329$, respectively).

Table 1. Clinical findings from peracute coliform mastitic cows (day 0 to day 3) and healthy cows

Parameters	Healthy cows (8)	Day 0 (11)	Day 2 (11)	Day 3 (11)
Rectal temperature (°C)	38.6 ± 0.2	39.1 ± 1.1	38.6 ± 0.2	38.7 ± 0.3
Heart rate (beats/min)	76.8 ± 3.7 ^{a,b}	103.4 ± 8.0 ^a	99.6 ± 8.1 ^b	95.4 ± 16.1
Respiration rate (enter unit here/min)	31.5 ± 2.6	33.3 ± 6.0	30.9 ± 5.6	29.1 ± 7.4
Clinical score	0 ^{c,d,e}	6.2 ± 1.3 ^{c,f,g}	3.8 ± 1.8 ^{d,f}	2.1 ± 2.4 ^{e,g}

Each value represents the mean ± SD for the number of experiments (n). Values with the same letter are significantly different at $P < 0.01$.

Table 2. Hematological values and number of somatic cells in milk of peracute coliform mastitic cows (day 0 to day 3) and healthy cows

Parameters	Healthy cows (8)	Day 0 (11)	Day 2 (11)	Day 3 (11)
Hematocrit values (%)	28.7 ± 1.4	32.7 ± 3.9	30.1 ± 4.6	29.4 ± 5.2
Leukocyte counts (/μl)	13,250 ± 5,742.8	6,136.4 ± 4,405.9 ^a	12,600 ± 9,948.3	15,845.4 ± 8,856.1 ^a
Platelet counts (×10 ⁴ /μl)	49.3 ± 3.5	39.0 ± 17.7	38.2 ± 16.6	39.9 ± 16.7
Total protein (g/dl)	7.35 ± 0.50	5.63 ± 1.87	5.91 ± 1.39	6.34 ± 1.77
Albumin (g/dl)	3.53 ± 0.13	2.71 ± 0.85	2.82 ± 0.67	2.96 ± 0.84
A/G (%)	0.90 ± 0.18	0.96 ± 0.20	0.94 ± 0.21	0.89 ± 0.17
Blood urea nitrogen (mg/dl)	10.8 ± 4.2 ^b	17.3 ± 4.3 ^{b,c}	14.1 ± 5.2	11.3 ± 2.1 ^c
Total cholesterol (mg/dl)	212.75 ± 53.72	139.91 ± 57.56	149.72 ± 72.57	151.36 ± 57.05
Total calcium (mmol/l)	2.48 ± 0.34 ^d	1.52 ± 0.55 ^d	1.87 ± 0.68	1.94 ± 0.59
Ionized calcium (mmol/l)	1.1 ± 0.1	0.98 ± 0.22	1.07 ± 0.16	1.07 ± 0.16
Inorganic phosphorus (mg/dl)	5.60 ± 2.05	3.47 ± 1.53	3.83 ± 1.56	4.52 ± 1.40
Magnesium (mg/dl)	2.16 ± 0.37	1.78 ± 0.56	1.71 ± 0.36	1.88 ± 0.56
Number of somatic cells in milk (×10 ³ /ml)	49.3 ± 3.5 ^{e,f,g}	25,638.1 ± 32,127.2 ^e	25,541.4 ± 35,022.5 ^f	10,270.3 ± 18,418.7 ^g

Each value represents the mean ± SD of the number of experiments (n). Values with the same letters are significantly different (e, f, and g are significant at $P < 0.01$ and a, b, c, d are at $P < 0.05$).

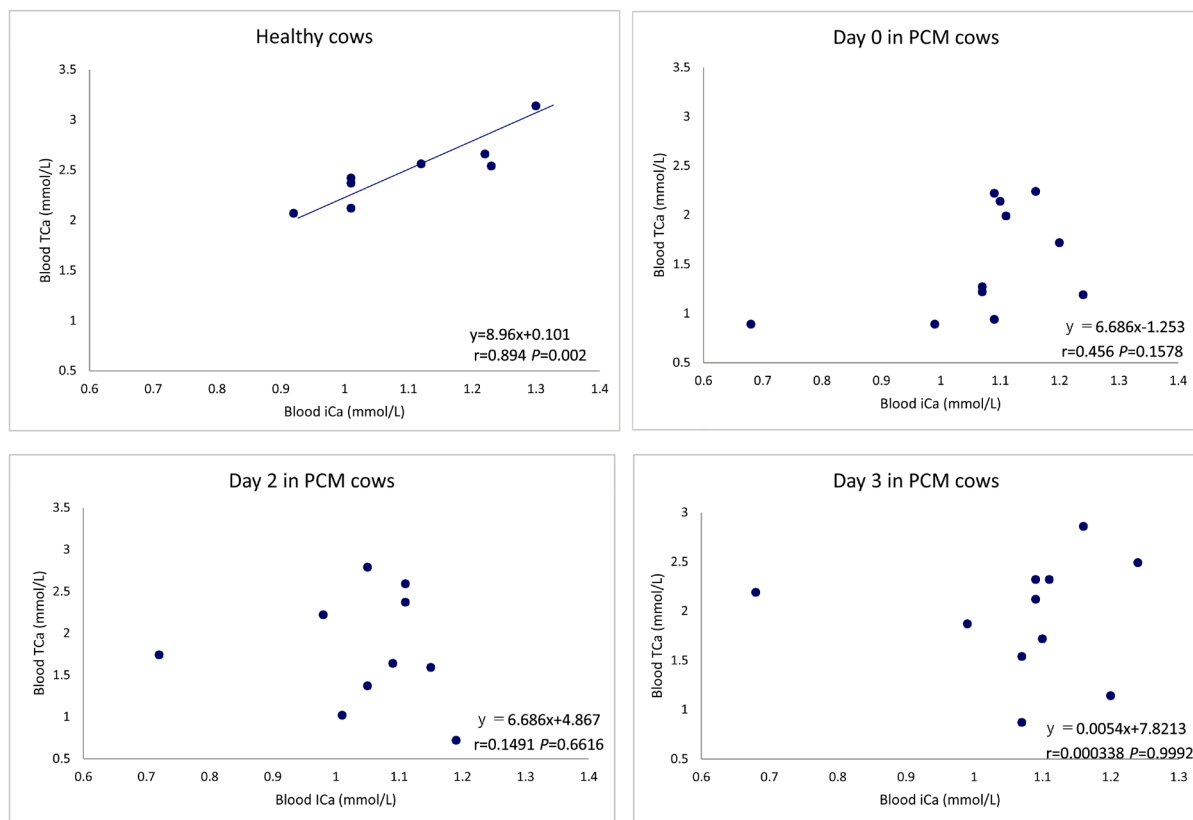


Fig. 1. Correlation between ionized calcium concentration (iCa) and total calcium concentration (TCa) in blood.

Table 3. Multiple regression analysis of ionized calcium concentration (iCa) concentration in the blood with clinical findings and hematological parameters for PCM cows at day 0

Summary of multiple regression analysis

Model	R	R ²	Flexibility decision finished multiple correlation coefficient	Adjusted R-square
1	0.9283	0.8618	0.8773	0.7697

Analysis of variance^{a)}

Model	Sum of squares	Degree of freedom	Unbiased variance	Variance ratio	Level of significance
Total variation	0.509	10			
Variation by regression	0.438	4	VR=0.11	F=9.356**	P=0.0095
Residual variation	0.07	6	VE=0.012		

Result of analysis

Explanatory variable	Coefficient ^{a)}			
	B ^{b)}	SE	β ^{c)}	r
(Constant)	0.0276	0.07		
Packed cell volume	-0.0347	1.196	-0.6108	-0.804
Platelet counts	0.0031	5.339	0.2413	0.6576
Rectal temperature	0.0451	0.323	0.2141	0.6182
Albumin	0.0745	0.256	0.2806	0.2842

Dependent variable is ionized Ca concentration in blood. Explanatory variable: packed cell volume, platelet counts, rectal temperature, albumin.

a) Dependent variable ionized Ca, b) partial regression coefficient, c) estimated regression coefficient. **, $P < 0.01$.

DISCUSSION

Blood ionized calcium has physiological activity and plays integral roles *in vivo*, including that in neurotransmitter release, muscle contraction, blood coagulation, and intracellular signal transduction [28]. It has been reported that healthy dairy cows exhibited hypocalcemia from 1 hr to 72 hr when lipopolysaccharide (LPS, 1,000 ng/kg) was experimentally injected intravenously [13]. When researchers injected LPS into the abdominal cavity of mice, hypocalcemia occurred because of endotoxaemia associated with a disruption of fibroblast growth factor 23-klotho-vitamin D signaling. [16]. In humans, hypocalcemia in critically ill patients with burn injuries or sepsis is associated with *CASR* gene-mediated up-regulation of tumor necrosis factor (TNF)- α and interleukin (IL)-1 β via NF- κ B elements, and by IL-6 via Stat1/3 and Sp1/3 elements in the *CASR* gene promoter, respectively [3, 10]. We have previously reported that the proinflammatory cytokines IL-1 β and IL-6 in the serum and whey of PCM cows at day 0 were significantly ($P<0.05$) higher than those in healthy cows [11]. The TCa concentrations of PCM cows at day 0 were significantly lower ($P<0.05$) than those of healthy cows, and iCa concentrations tended to be lower. Therefore, hypocalcemia of PCM cows at day 0 was considered to be associated with the production of inflammatory cytokines.

It has been reported that experimental intravenous injection of LPS (2 μ g/kg) in healthy dogs reduced serum iCa, TCa, and vitamin D levels and increase the PTH concentration [12]. In this study, a positive correlation was detected between iCa and TCa levels in healthy cows, but no correlation was found from day 0 to day 3 for PCM cows. This may have occurred because of the production of inflammatory cytokines in PCM cows, which decreases PTH secretion and the conversion from 25-OH vitamin D to 1,25-D [3, 9, 10].

Ohtuka *et al.* reported that severe cases of coliform mastitis in cows exhibited marked clinical signs, such as loss of appetite, depression of digestive tract motility, and metabolic alkalosis, including an increase in blood pH, hypochloremia, and hypokalemia compared with that of mild cases and healthy cows [17]. In humans, severely ill patients with hypocalcemia, exhibit alkalization of blood pH (pH ≥ 7.45), which may increase the binding of iCa to proteins, and consequently decrease blood iCa [5]. Hagiwara *et al.* reported that decreased platelet counts and increased packed cell volumes are important parameters for determining the prognosis in acute coliform mastitis in cows [8]. TCa concentration in the blood of PCM cows at day 0 was not related to any other value. However, we found a positive correlation between iCa concentration in the blood of PCM cows at day 0 and rectal temperature, platelet counts, and albumin levels and a negative correlation between iCa and hematocrit values using multiple regression analysis ($r=0.6182$, $r=0.6576$, $r=0.284$, $r=-0.804$, $P<0.01$, respectively). Thus, we determined that low iCa concentration in the blood of PCM cows at day 0 was related to symptoms of septic shock, such as hypothermia, blood coagulation system activation, and dehydration.

In this study, one PCM cow died of septic shock without improvement in clinical signs on the 5th day. All 10 PCM cows that recovered showed improved systemic signs, and at the end of 2 weeks, exhibited no swelling or induration of the mastitic quarter. The cows that recovered produced the same milk yield as they did prior to day 0. In our previous report [11], PCM caused by *K. pneumoniae* was severely symptomatic and ceased lactation in mastitic quarter. Reports indicated that administration of enrofloxacin as treatment of acute mastitis caused by *E. coli* improved appetite and lowered the number of somatic cells in the mastitic quarter after treatment [19, 22]. In our previous report [11], we used kanamycin or cefazolin as antibiotics. We speculate that the administration of enrofloxacin and vaccination [2, 27] affected the recovery of PCM cows.

In children, 77.1% of ionized hypocalcemia was observed early in sepsis because of being of their being in an intensive care unit [6]. In conclusion, immediate measurement of blood iCa levels on day 0 with a portable calcium ionization meter may indicate the severity of septic shock caused by this disease. In the future, the authors will further investigate the immunological relationships of blood iCa in PCM cows.

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