Article

Reference limits for biochemical and hematological analytes of dairy cows one week before and one week after parturition

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Abstract — Since dairy cows during the transition period have multiple endocrine and metabolic changes, it is necessary to determine the reference limits of laboratory analytes in normal transition cows. Reference limits for the weeks before and after calving were determined in dairy cows. Animals that had adverse clinical outcomes after calving and cows that were culled or had mastitis within the first 7 days after calving were excluded. All biochemical analytes (β-hydroxybutyrate, fatty acids, glucose, cholesterol, urea, calcium, and phosphorus) were statistically different between precalving and postcalving groups. The hematological analytes were not significantly different except for eosinophils. The data from precalving and postcalving cows were significantly different from reference limits in a university-associated laboratory derived from early- and mid-lactation cows. Different reference limits for precalving and postcalving dairy cows should be determined for biochemical analytes to ensure appropriate interpretation of results.

Résumé — Limites de référence pour les paramètres biochimiques et hématologiques des vaches laitières une semaine avant et une semaine après la parturition. Vu que, durant la période de transition, il y a plusieurs changements endocriniens et métaboliques chez les vaches laitières, il est nécessaire de déterminer les limites de référence pour les paramètres de laboratoire chez les vaches à transition normale. Les limites de référence pour les semaines avant et après le vêlage ont été déterminées chez les vaches laitières. Les animaux qui étaient dans un état clinique indésirable après le vêlage et les vaches qui ont été éliminées ou avaient une mammite pendant les 7 premiers jours après le vêlage ont été exclus. Tous les paramètres (β-hydroxybutyrate, acides gras, glucose, cholestérol, urée, calcium et phosphore) étaient statistiquement différents entre les groupes avant et après le vêlage. Les paramètres hématologiques n'étaient pas significativement différents, à l'exception des eosinophiles. Les données des vaches avant et après le vêlage étaient significativement différentes par rapport aux limites de référence d'un laboratoire affilié à une université qui étaient dérivées de vaches au début et au milieu de la lactation. Des limites de référence différentes pour les vaches laitières avant et après le vêlage devraient être déterminées pour les paramètres biochimiques afin de garantir une interprétation appropriée des résultats.

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Introduction

During the dairy cow production cycle, the transition period is critical due to the tumultuous endocrine and metabolic changes that accompany parturition and the initiation of lactation (1–4). Laboratory medicine is an important tool that helps practitioners monitor transition cow health at the individual and herd levels (5–8). Through laboratory profiling, not only can sick animals be detected, but those herds with higher risks for developing metabolic, reproductive, or infectious diseases may be predicted (9–14).

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Any measurement that may be used to make a decision about an intervention must be compared with an appropriate reference value, or more precisely, an interval of reference value (15). Reference limits should be determined by each laboratory under their specific conditions of equipment, reagents, target population, and staff training. Alternatives to these reference limits are those provided by the reagent manufacturers, reference laboratories working in similar conditions, or textbooks (16). However, these alternatives should be used with considerable caution.

Several methodologies have been described to establish reference limits (17,18). However, the data that are the basis of the reference limits, including the sample size and the variable distribution, should be carefully considered (15,19). The smaller the sample, the wider the confidence interval, and consequently, more caution must be applied when using them to interpret results. A sample size of 60 is proposed to be sufficient to establish reference limits for domestic animals (20). If the

variable does not fit the normal distribution, adjustments such as logarithmic or squared transformations are possible tools to normalize the data to calculate valid descriptive statistics (19).

In veterinary medicine, a pragmatic approach must be applied due to the diversity of species and complexity in how they can be subgrouped. However, it is necessary to understand the characteristics of the reference population that is to be sampled in order to most accurately interpret the results. Due to the changes that take place during the transition period, it is necessary to determine different reference limits for both the precalving and postcalving periods. The objective of this study was to establish reference limits for dairy cows in the week prior to calving and the week after calving, for several commonly measured biochemical and hematological analytes.

Materials and methods

The present study was based on data originally used in previous field studies (14,21). From September 1998 through October 1999, 20 dairy farms in southwestern Ontario were visited weekly, on the same day of the week for each herd, at approximately the same time, within 2 h of the morning feeding. Cows (n = 1072) within 15 d before the predicted calving day, were sampled weekly until 15 d in milk. The calving date was recorded. The profiles from cows between 1 and 8 d before calving were used to determine the precalving reference limits. The profiles from cows between 0 and 7 d after parturition were used to establish the postcalving reference limits.

Body condition was scored at the first visit. Signalment data including herd, breed, and lactation were recorded. Twin calving and clinical problems such as udder edema, dystocia, retained placenta, metritis, milk fever, ketosis, abomasal displacement, off-feed, lameness, and clinical mastitis or culling before 7 d in milk (DIM), were recorded as dichotomous variables. Cows with any of the preceding clinical outcomes, except for twin calving, were excluded. Cows with clinical mastitis or that were culled after day 7 postcalving were included, because these outcomes are not necessarily related to parturition.

Blood from the coccygeal vessels was collected in 10-mL evacuated plain tubes and 5-mL tubes containing ethylenediamine tetra-acetic acid (EDTA) anticoagulant (Vacutainer; Becton-Dickinson, Franklin Lakes, New Jersey, USA). Samples from plain tubes were allowed to clot for 30 to 60 min, then centrifuged for 10 min at 1300 × g. Subsequently, the serum was harvested, and stored at -20°C until analysis. Unclotted blood samples were kept chilled until analysis within 8 h. The samples were submitted to the Animal Health Laboratory (AHL), University of Guelph, for processing.

The following biochemical analytes were determined in serum, using colorimetric methods in an automated spectrophotometric chemistry analyzer (Hitachi 911; Roche, Tokyo, Japan). Interassay coefficients of variation are included in brackets. Fatty acids [3.2%] (NEFA; Randox laboratories, Antrion, United Kingdom), β-hydroxybutyrate [8.1%] (Ranbut; Randox laboratories), cholesterol [1.9%] (Roche diagnostics GMbH, Manheim, Germany), glucose [1.5%] (Roche diagnostics GMbH), urea [2.3%] (Roche diagnostics GMbH), calcium [2.8%] (Roche diagnostics GMbH), and phosphorus [1.8%] (Roche diagnostics GMbH). Total leukocytes, neutrophils, lymphocytes, monocytes and eosinophils were determined on an automated hematological analyzer (Technicon H1; Bayer, Toronto, Ontario).

Calculation of reference limits

Descriptive statistics, mean and median, and reference limits, including the respective 95% confidence intervals (CI) were determined using a commercial software program (Analyse-It software, version 1.71, 2003). Comparisons between precalving and postcalving cows and parity groups within each period were done with a Generalized Linear Model (GLM) and Tukey test, using SAS (Statistical Analysis System [SAS] Version 9.1; SAS Institute, Cary, North Carolina, USA). Within precalving and postcalving periods, cows were subdivided into 1st (Lac1), 2nd (Lac2) and 3rd or greater lactation (Lac3+); reference limits were determined and groups were compared. The level of significance was set at $P = 0.05$. The decision to use parametric or nonparametric tests to establish the reference limits was based on the test of normal distribution of the analyte data, using the Shapiro-Wilk coefficient of ≥ 0.95. Biochemical analyte results were compared with those currently used by the AHL. The current reference limits are based on 90 cows in different stages of lactation, approximately 70% of which were cows between 30 and 100 d in milk (Brent Hoff, personal communication, 2007). The reference limits used by the AHL were determined using the same analyzer used in the present study. A GLM was used to compare the means and the Kolmogorov-Smirnov test was used to compare the distribution curves.

Results

There were significant differences in the comparison of means of the biochemical analytes [β-hydroxybutyrate (BHB), fatty acids, glucose, cholesterol, urea, calcium, and phosphorus] between the precalving and postcalving periods ($P < 0.001$). The means of the hematological analytes were not statistical different over the study period ($P > 0.05$) except for the numbers of eosinophils ($P < 0.001$). The most marked variations between the precalving and postcalving values were observed in the BHB mean, median and upper limit; the fatty acid mean, median, and upper limit; all calcium parameters; all phosphorus parameters; leukocyte lower and upper limits; neutrophil lower and upper limits; and eosinophil mean, median and upper limit.

Through the examination of groups by lactation, BHB showed a significantly lower mean at Lac2 compared to Lac3+ in the postcalving period. Fatty acid concentrations were statistically different in all groups precalving and postcalving except between Lac1 and Lac3+ in postparturient cows. Cholesterol was significantly lower in Lac2 compared with Lac3+ in the precalving period, but not between other comparisons. Glucose concentrations were different except between Lac2 and Lac3+ in the precalving period. Urea was significantly lower in Lac1 compared with Lac2 as well as compared with Lac3+ in both pre- and post-calving groups. Calcium concentrations were significantly different except for Lac2 and Lac3+ in the precalving period. Phosphorus was not significantly different in all precalving comparisons. Furthermore, phosphorus values were

Table 1. Differences of means of biochemical and hematological analytes between lactation groups in dairy cows 1 week before calving and 1 week after calving

Analyte	Precalving cows	Postcalving cows
β-hydroxybutyrate	NSD	L2 < L3+
Fatty acids	L2 < L3+ < L1	L2 < L1; L2 < L3+
Cholesterol	L2 < L3+	NSD
Glucose	L2 < L1, L3+ < L1	L2 < L3+ < L1
Urea	L1 < L2; L1 < L3	L1 < L2; L1 < L3
Calcium	L2 < L1, L3+ < L1	L3+ < L2 < L1
Phosphorus	NSD	L2 < L1, L3+ < L1
Total leukocytes	L3+ < L1; L3+ < L2	L3+ < L1
Neutrophils	L3+ < L1; L3+ < L2	L3+ < L1
Lymphocytes	L3+ < L1	NSD
Monocytes	NSD	NSD
Eosinophils	L1 < L2	L1 < L2

Significance *P*-value = 0.05.
Precalving cows: Interval between days -8 and -1 of calving.
Postcalving cows: Interval between days 0 and 7 of calving.
L1 — Cows in first lactation.
L2 — Cows in second lactation.
L3+ — Cows in third or greater lactation.
NSD — Nonsignificant differences.

significantly higher in Lac1 compared to Lac2 and Lac3+ in the postcalving period. Total leukocytes and neutrophils were statistically different in both precalving and postcalving periods when comparing the Lac1 and Lac3+ groups, as well as between Lac2 and Lac3+ in the precalving period. Lymphocytes were significantly higher in the Lac1 compared with Lac3+ in the precalving period. There were no significant differences in monocyte numbers. Eosinophils were statistically lower in both periods when comparing Lac1 with Lac2. These differences of means are summarized in Table 1.

Both precalving and postcalving values were significantly different from the current reference limits for the AHL ($P < 0.001$). When comparing the means between the precalving or postcalving period and the AHL group separately, significant differences ($P < 0.01$) were obtained, with the exception of some specific cases. Differences were not found with BHB postcalving versus AHL ($P = 0.32$); urea precalving versus AHL ($P = 0.12$), and urea postcalving versus AHL ($P = 0.42$); glucose postcalving versus AHL ($P = 0.86$); and phosphorus precalving versus AHL ($P = 0.09$). Significant differences were observed in the distribution curves when comparing the precalving or postcalving period and the AHL group separately ($P < 0.01$), except in the following cases: BHB postcalving versus AHL ($P = 0.98$); urea precalving versus AHL ($P = 0.37$); glucose postcalving versus AHL ($P = 0.17$); and phosphorus, both precalving and postcalving versus AHL ($P = 0.23$). Additionally, the difference for urea between the postcalving and AHL groups had a *P*-value of 0.04. The reference limits for the biochemistry and hematology analytes are summarized in Tables 2 and 3, respectively.

Discussion

Many studies have reported differences in reference limits for cattle, associated with age, gender, breed, or production purpose (dairy or beef) (22–26). Serum glucose was higher in suckling calves compared with 1-year-old calves, and serum protein was lower in heifers compared to older cows (24,25). However, none of the cited reports described precalving and postcalving reference limits during the transition period.

It is well known that there are profound physiological changes in certain analytes between the precalving and postcalving periods. These changes are not necessarily indicative of disease but reflect physiological variations. As an example, calcium drops in the 2–3 d around calving due to the onset of colostrum/milk production (27). The cows that were included in the present study did not have clinical disease during the first 30 d after calving. Those that had mastitis or were culled after the 7th day were included because it was impossible to attribute the cause of such infection or the disease resulting in culling directly to the periparturient period or situations related exclusively to the lactation period itself.

Reference limits of biochemical analytes developed in the present study to define transition cows differ from those used by the AHL. The AHL reference limits were derived from 90 mid-lactation cows; approximately 70% of those were between 30 and 100 d in milk. Since preparturient disease is common in dairy cattle, it may be inappropriate to use mid-lactation cow limits in the interpretation of transition cow data. Interpretation of laboratory data from the transition cow using peak/mid-lactation reference limits may result in false conclusions and improper and/or unnecessary interventions. It was not possible to conduct a formal statistical comparison of the hematological data between the limits obtained in the present study and those used by the AHL to obtain the current reference limits because the data no longer existed in the database. Through an observational review of the respective limits, these appear similar to each other.

Expected BHB and fatty acid increases are associated with changes in energy demand and reduction in the dry matter intake with consequent lipid mobilization (28,29). The 1.0 mmol/L determined as the upper limit for fatty acids in precalving cows in the present study is almost double the cut-point used as predictors of negative energy balance (NEB) by other researchers. These authors reported that cows with high concentrations of fatty acids during the precalving period are predisposed to metabolic or infectious diseases (10,30). LeBlanc et al (14) reported a 3.6 times greater likelihood of left displaced abomasum (LDA) in cows that had ≥ 0.5 mmol/L of fatty acids between 0 and 6 d before calving. Cameron et al (10) reported a cut-point of > 300 μEq/L (0.3 mmol/L) of fatty acids between 35 d before calving and 3 d after parturition, but they did not specify a likelihood value or odds ratio. Cut-points are distinct from reference limits. Cut-points are used to predict the likelihood of a certain event and do not necessarily mean that a cow will develop that outcome. The Texas Veterinary Medical Diagnostic Laboratory (TVMDL) has different reference limits for dairy cows according to production stage; they use the upper limit of 0.35 mmol/L of fatty acids in transitional cows (Robert Sprowls 2006, personal communication). To our knowledge, there are no other laboratories currently using differential reference limits. None of the cows used in the present study had clinical problems after calving. The upper limit of the reference range is somewhat higher than all literature reports which recommend limits > 0.5 mmol/L to predict NEB, either as a cut-point (7,10,14) or a reference limit (TVMDL). The discrepancy between the results in this study and those

Table 2. Comparison of clinical chemistry reference limits currently used at the Animal Health Laboratory (AHL) and those determined in pre- and post-calving cows in southern Ontario dairy farms

Analyte	Unit	AHL[a]		Precalving cows			Postcalving cows		
β-hydroxybutyrate	μmol/L	324–1296	a	218–884	(498)[b]	b	216–1177	(527)[b]	a
			A			B			A
Fatty acids	mmol/L	0.1–0.4	a	0.0–1.0	(504)	b	0.1–1.4	(535)	c
			A			B			C
Cholesterol	mmol/L	1.7–7.7	a	1.3–3.0	(497)	b	1.9–2.9	(528)	c
			A			B			C
Glucose	mmol/L	2.5–4.3	a	2.64–4.75	(496)	b	2.3–5.2	(528)	b
			A			A			B
Urea	mmol/L	3.0–8.0	a	2.1–8.0	(499)	b	1.9–7.8	(525)	b
			A			A			B
Calcium	mmol/L	2.10–2.80	a	2.18–2.65	(511)	b	1.64–2.61	(527)	a,b
			A			B			C
Phosphorus	mmol/L	1.47–2.63	a	1.48–2.65	(347)	b	1.04–2.73	(554)	a,b
			A			B			B

[a] Biochemical values obtained from 90 clinically healthy cows, 50% first lactation, all milking 30–150 d, 10 Ontario farms.
[b] Sample size is included in parenthesis.
Different lowercase letters in the same row indicate statistical difference of means by the GLM test ($P < 0.05$).
Different uppercase letters in the same row indicate statistical difference of distribution curves by the Kolmogorov-Smirnov test ($P < 0.05$).

Table 3. Comparison of hematological reference limits currently used at the Animal Health Laboratory (AHL) and those determined in pre- and post-calving cows in southern Ontario dairy farms

Analyte	Unit	AHL[a]	Precalving cows		Postcalving cows	
Leukocytes	$\times 10^9$/L	5.0–13.3	4.8–14.6	(485)[b]	3.9–17.0	(514)[b]
Neutrophils	$\times 10^9$/L	1.7–6.0	1.7–6.2	(490)	0.7–9.0	(518)
Lymphocytes	$\times 10^9$/L	1.8–8.1	1.9–9.0	(475)	2.1–10.0	(507)
Monocytes	$\times 10^9$/L	0.1–0.7	0.0–0.3	(485)	0.0–0.3	(536)
Eosinophils	$\times 10^9$/L	0.1–1.1	0.1–1.2	(499)	0.0–0.7	(536)

[a] Hematological values obtained from 90 clinically healthy cows, 50% first lactation, all milking 30–150 d, 10 Ontario farms.
[b] Sample size is included in parenthesis.
Statistical comparison between groups was not performed due to inaccessibility of raw data from the AHL. Reference limits were determined with different instruments.

in the literature suggests that further work needs to be done to determine the correct threshold value for interpretation of fatty acids in the precalving period, which will depend on the objective of the sampling.

The postcalving BHB upper limit was 1177 mmol/L. This value is very close to the cut-point of 1200 mmol/L that LeBlanc et al (14) found associated with an 8-times risk of LDA in postparturient cows. Cows with results close to the upper limit for BHB are likely at higher risk of LDA.

The reduction of serum cholesterol is related to the switch in synthesis of lipoproteins from the hepatocytes. In the postpartum period there is an increase in circulating high density lipoproteins and a drastic decrease in low density lipoproteins (LDL), as well as reductions in very low density lipoproteins. Low density lipoproteins contain the largest proportion of cholesterol amongst the lipoproteins (31). The determined interval is narrower than that currently used by the AHL; however, it is similar to that reported by Kaneko et al (32). The narrower limits may reflect the larger sample size used in the present study in contrast to the sample size used by the AHL.

Compared with other reported reference limits, those for glucose had wider intervals with a higher upper limit in the present study. However, other intervals are based on peak/mid-lactation cows and/or beef cattle (22,24,25,32). The exception is the reference limit used at the TVMDL (3.19 ± 0.38 mmol/L), which is very narrow and low. During the transition period, several hormonal changes take place, primarily to regulate parturition and initiate lactation, and secondarily to adapt metabolism to those events (33,34). These mechanisms produce a hypoglycemic state after parturition (35). However, it is possible that some cows exhibit a gluconeogenic effect of epinephrine and cortisol because of the excitement and stress associated with parturition.

During handling of the samples, efforts were made to ensure rapid separation of serum from the cells, but this could not always be done promptly. Therefore, it is possible that some samples could have had an artifactually low glucose concentration due to in vitro consumption. Given the large sample-size, however, the significance of this change might be minimal. It is difficult to accurately determine the impact of this possible mishandling in the final reference limits for glucose. Unfortunately it is not possible to discriminate samples according to handling to better interpret its influence in the limits obtained.

Urea is an important source of protein synthesis in ruminants through the urea cycle (7,36). Urea in ruminants is synthesized in the liver from 2 sources: nitrogen from the deamination of endogenous amino acids and ammonia absorbed from the rumen (37). During lactation, the udder uses large amounts of protein for milk production (38). Reduced dry matter intake might cause a drop in the ammonia absorption, causing a shift of urea being recycled in the rumen, which may explain the lower serum concentrations of urea in the postparturient period.

A well-recognized pathophysiological event in dairy cows during the transition period is the drop in serum calcium

concentrations that occurs at parturition or in the 1st days after calving (39–42). This reduction in calcium is caused by the onset of lactation, where an important loss of calcium in colostrum and milk occurs (40). It is known that older cows will have lower concentrations of calcium (43). One of the reasons is decreased numbers of receptors for 1,25-dihydroxyvitamin D in the intestine, resulting in decreased absorption of the mineral (40). This situation might explain the very low lower limit determined for calcium (44,45).

No significant differences in leukocytes were observed except for a decrease in eosinophils after calving. This change was previously reported and may result from the stress (cortisol mediation) associated with parturition (46). Other typical changes of acute stress in cows like neutropenia or lymphopenia (47) were not observed.

The limits obtained in the present study for both precalving and postcalving samples differed from those currently used by the AHL for all biochemical analytes except urea.

Different reference limits for precalving and postcalving dairy cows should be determined for biochemical analytes, in order to permit clinicians to make appropriate interpretation of the results. Particular attention should be paid to BHB, fatty acids, and calcium, in which the determined limits for the weeks before and after calving are markedly different from those for peak- or mid-lactation reference limits. Hematological differences were not critical; peak- or mid-lactation reference limits are likely applicable to the transition period cows.

Marked biochemical changes were present around the transition period. It is clear that misinterpretation could occur if inappropriate reference limits are used. Clinicians need to understand that interpreting the laboratory results should be done in conjunction with complementary information (reproductive performance, milk yield, etc.), including physical examination data.

Authors' contributions

Drs. Quiroz-Rocha, LeBlanc, Duffield, Leslie, and Jacobs designed the experiment, analyzed the data, and wrote the manuscript. Drs. LeBlanc and Duffield obtained the samples and compiled the results. Dr. Wood supervised the analytical methodology and reviewed, with Dr. Jacobs, the statistical procedures.

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Book Review
Compte rendu de livre

Your Cat — A Revolutionary Approach to Feline Health and Happiness

Hodgkins EM. Thomas Dunne Books, New York, New York, 2007. ISBN 0-3123-5801-6. US$27.95.

This easy-to-read book for the cat enthusiast is written to "raise the alarm" regarding flaws in commercial diets fed today. The author attributes feline inflammatory cystitis, kidney failure, hyperthyroidism, as well as inflammatory bowel disease to mistakes in diet formulation.

The author's credentials include being a veterinarian since 1977, as well as a cat breeder. She also presents facts and opinions gleaned through her involvement in the pet health insurance industry and as an executive for a pet food manufacturer.

Speaking on nutrition, Hodgkins suggests that cats have a low thirst drive. Feeding dry food creates concentrated urine with resultant medical problems. She eliminates all dry foods from consideration when choosing a food for cats.

When contemplating food ingredients, the author calls for a stop to feeding "foods meant more for fattening cattle than nourishing a top predator." Her answer is a high protein, low carbohydrate food provided by either a canned commercial preparation or a raw meat diet.

As well, the author suggests that pet foods are not tested sufficiently prior to being sold and pets are being used as unknowing victims in ongoing food experiments. She thinks that veterinarians are seeing chronic diseases resulting from inadequate diets, but they haven't made the connection between disease and inferior nutrition.

There are very detailed chapters on how to battle obesity as well as managing chronic renal failure. The recommendations make sense based on Hodgkin's assumptions. Many other diseases commonly seen in cats are also explained for the lay person.

The author advocates keeping cats indoors for their own safety, but this could be due to the area where she resides. There are also some ideas introduced for keeping cats socially happy and how to avoid behaviors that owners find annoying.

The over-riding goals of the book are to assist cat owners in taking better care of their cat and to look at the current recommendations critically. The facts presented by Hodgkins can be taken in one of two ways. Some veterinarians would consider her views as opinion unsubstantiated by fact. The opposing view is that she presents progressive material that will be proven over time.

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