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Short communication: Reference values for blood parameters in Holstein dairy cows: Effects of parity, stage of lactation, and season of production

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ABSTRACT

Confidence intervals for blood parameters used for nutritional and metabolic profile testing in cattle were calculated for clinically normal lactating Holstein cows, taking into account the effects of parity, stage of lactation, and season. Blood samples were collected from 740 cows in 33 Italian dairy herds according to a predefined protocol. Herds were visited during summer and the following winter, sampling 12 lactating cows at each visit (4 primiparous and 8 multiparous). Six cows were selected from the early-lactation group (days in milk: 10 to 89) and the other 6 were selected from the mid-lactation group (days in milk: 90 to 215). Cow selection criteria excluded animals clinically exposed to periparturient diseases as well as animals not considered in good health by a veterinary clinical examination. For each blood variable, outliers were identified and discarded. Data were then analyzed for their Gaussian distribution and variables with not normal distribution were log-transformed to adjust for lack of normality. Herd mean values were calculated for each blood parameter according to 3 main classification factors: parity (primiparous vs. multiparous), stage of lactation (early vs. mid) and season of production (summer vs. winter). The resulting data set was statistically analyzed using a mixed model with the fixed effects of these factors, their interactions, and the random effect of herd. General 95% confidence intervals were calculated for blood variables that showed a relevant herd variance component such as albumin, triglycerides, aspartate, urea, glucose, alanine aminotransferase, lactate dehydrogenase, direct and total bilirubin, calcium, magnesium, and potassium. For the remaining parameters, specific confidence intervals were calculated for each level of the significant main factors. Parity affected blood concentration of total protein, globulin, creatinine, alkaline phosphatase, gamma glutamyl transferase, creatinine kinase, and phosphorus. Blood nonesterified fatty acids, aspartate aminotransferase, gamma glutamyl transferase, creatinine kinase and cholesterol were influenced by stage of lactation. The season of production had a significant effect on total protein, globulin, creatinine, alkaline phosphatase, phosphorus, sodium, and chlorine. The outcomes of this work will improve the accuracy of the biochemical profile as a tool for dairy practitioners to assess the metabolic status of lactating Holstein cows.
Key words: blood reference value, Holstein cow, parity, season of production

Short Communication

Laboratory medicine parameters are an important tool that helps dairy practitioners to monitor cow health at the individual and herd level. To identify anomalous situations in a given dairy herd, values from blood analysis are generally compared with the population average or ranges of standard values (Herdt, 2000). However, many of these published reference intervals have been established for cattle and do not distinguish between dairy and beef animals (Russell and Roussel, 2007; Kaneko et al., 2008). When available for dairy cows, they have been generated from a small number of cows (Moore, 1997) or only for a specific physiological phase like the transition period (Quiroz-Rocha et al., 2009; Ospina et al., 2010).

It is known that milk production is affected by factors such as parity, stage of lactation, and season of production. The same factors might have some effect also on blood parameters in clinically normal lactating cows. Therefore, the calculation of specific confidence intervals according to these relevant factors could improve the effectiveness of blood proofing to detect abnormalities in a given dairy breed. This study was designed to produce confidence intervals for metabolic profile testing of clinically normal lactating Holstein cows, taking into account parity, stage of lactation, and season.

The study was carried out in the eastern part of the Po Valley in Italy where over 70% of the 1.1 million cows belonging to the Italian Holstein population are raised. This cattle population has a strong genetic relation for

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dairy production traits with American and Canadian Holsteins ($r = 0.94$ and 0.93, respectively; Interbull, 2010). Farm selection was performed to choose herds with good production performance and low incidence of nutritionally related problems of disease. Criteria for farm inclusion in the sample were herd size (>30 lactating cows), rolling milk yield (>8,000 kg of milk), culling rate (<30%), days open (<150) and bulk tank SCC (<300,000/mL). Additionally, to limit the effect of the different feeding regimens, only farms feeding TMR were considered. From the total number of farms that met the previous criteria, a convenience sample was created on the basis of the farmers' willingness to be part of the study and the presence of a farm veterinarian available for clinical visits and blood sample collection. Thirty-three dairy herds were included in the farm sample (8% of the total Holstein herds of the area). Herds were visited during the summer (August to September) and the following winter (January to March). Twelve lactating cows were sampled per herd at each visit (4 primiparous and 8 multiparous). Six cows were selected from the early-lactation group (accepted range: from 10 to 89 DIM) and the other 6 were selected from the mid-lactation group (accepted range: from 90 to 215 DIM). Cow selection criteria excluded animals clinically exposed to periparturient diseases, as well as animals not considered healthy by a veterinary clinical examination. This clinical visit was carried out on the day before blood sampling and it considered the following assessments: body temperature (accepted range: 38.0 to 39.0°C); rumination activity (≥60 chews/cud); fecal score using the method proposed by Hutjens (2002; accepted score range: 2 to 3); urine analysis by Combur-Test strips (Roche Diagnostics S.p.A., Milan, Italy); pH (accepted range: 8.2 to 8.4); ketones (absence); proteins (absence); and udder health by the California Mastitis Test (Immucell Corp., Portland, ME; negative). Individual blood samples were collected in the morning, before TMR distribution. Samples were taken from the jugular vein into vacuum tubes containing 150 USP units of lithium heparin (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ). At the laboratory, blood samples were centrifuged at 1,500 × *g* for 15 min at 4°C and plasma was stored at −18°C. From the 792 samples collected according to the research protocol, 52 samples were discarded due to hemolysis or to incorrect storage and handling before analysis. The final analyzed sample set considered 372 summer versus 368 winter samples, 231 primiparous versus 509 multiparous cows, and 355 early- versus 385 mid-lactating cows. A set of parameters routinely used by dairy practitioners for blood profiling of lactating cows were considered in the study: protein and energy metabolism parameters (total protein, globulin, albumin, urea, creatinine, glucose, NEFA, and triglycerides); enzymes and hepatic markers [alanine aminotransferase, aspartate aminotransferase (**AST**), alkaline phosphatase (**ALP**), gamma glutamyl transferase (**GGT**), lactate dehydrogenase, creatinine kinase (**CK**), cholesterol, and direct and total bilirubins]; and minerals (calcium, phosphorus, magnesium, sodium, potassium, and chloride). All plasma samples were analyzed on the Hitachi 911 (F. Hoffmann-La Roche Ltd., Basel, Switzerland) at 37°C. Roche BM commercial kits were used, except for the concentration of NEFA in plasma, which was measured with the enzymatic colorimetric method (Randox Laboratories Ltd., Crumlin, UK). The analytical method of each parameter and the corresponding quality laboratory assay are reported in Table 1.

The statistical procedure used to calculate confidence intervals for the different blood parameters followed the recommendation of the International Federation of Clinical Chemistry (Solberg, 1987). The first step was the detection of outlier data for each blood variable and values more than 3 standard deviations away from the mean were discarded. Data were then analyzed for their Gaussian distribution and coefficients of skewness and kurtosis were computed by PROC UNIVARIATE (SAS 9.2; SAS Institute Inc., Cary, NC) to measure the distribution asymmetry and peakedness. Variables with Shapiro-Wilk values (W) ≥ 0.98 were considered normal, whereas all other variables were log-transformed before analysis to adjust their lack of normality. As suggested by Herdt (2000), mean values for each blood parameter were calculated within herd for each main factor: parity (primiparous vs. multiparous), stage of lactation (early vs. mid) and season (summer vs. winter). This herd-level approach has the advantage of decreasing variability, as the standard deviation of a herd means the population will always be lower than the standard deviation of values from a population of individual cows. Gaussian and transformed data were statistically processed by PROC MIXED (SAS 9.2; SAS Institute Inc.) using a linear model to assess the fixed effects of parity, phase of lactation, season, their interactions, and the random effect of herd. Reference values for blood parameters were generated based on the significant outcomes of the model. A single general 95% confidence interval was calculated for parameters that showed a herd variance component greater than 30% of the total variance or when no fixed effects or interactions reached the minimum threshold of statistical significance ($P < 0.01$). For the remaining parameters, specific confidence intervals were calculated for each level of a given significant factor (parity, stage of lactation, season of production, and their interactions). For the non-normal distributed variables, means and confidence intervals were calculated on the log-transformed

Table 1. Blood parameters, analytical methods, and quality laboratory assays

Parameter[1]	Analytical method	Intra-assay variation (CV%)	Inter-assay variation (CV%)
Protein and energy markers			
Total protein	Biuret reaction	0.65	0.95
Globulin	Calculated parameter	—	—
Albumin	Albumin bromcresol green (BCG) method	1.41	1.99
Urea	UV kinetic (urease and glutamate dehydrogenase GLDH)	2.08	3.4
Creatinine	Enzymatic; prostatic acid phosphatase (PAP)	1.43	2.01
Glucose	Hexokinase, G6PDH	1.22	1.70
NEFA	Colorimetric		
Triglyceride	Enzymatic; glycerol-3-phosphate oxidase (GPO)-PAP	4.17	4.20
Enzymes and hepatic markers			
ALT	IFCC[2] 37°C without pyridoxal-5-phosphate (P5′P)	4.40	4.4
AST	IFCC 37°C without P5′P	1.25	1.65
ALP	IFCC 37°C liquid	0.58	0.67
GGT	Standardized liquid Szasz	1.5	1.9
LDH	Optimized Deutsche Gesellschaft für klinische Chemie (DGKC)	1.94	2.60
CK	IFCC 37°C	1.11	1.40
Cholesterol	Enzymatic: cholesterol oxidase (CHOD)-PAP	2.08	2.56
Direct bilirubin	Jendrassik	1.22	1.74
Total bilirubin	N,N-diethyl-p-phenylenediamine (DPD)	3.32	5.46
Mineral			
Calcium	Colorimetric: *o*-cresolphtalein complexone	1.02	1.63
Phosphorus	Phosphomolybdate	0.77	1.41
Magnesium	Xylidyl blue method	1.30	2.83
Sodium	Indirect potentiometry	0.48	0.47
Potassium	Indirect potentiometry	0.37	0.79
Chlorine	Indirect potentiometry	0.31	0.99

[1]ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; GGT = gamma glutamyl transferase; LDH = lactate dehydrogenase; CK = creatinine kinase.

[2]International Federation of Clinical Chemistry and Laboratory Medicine.

values and the results were then reported in tables after antilog transformation.

Only a limited number of outliers were detected and the parameters with the highest number of discarded values were NEFA and AST (Table 2). The assessment of the Gaussian distribution showed anomalous skewness and kurtosis coefficients and W values below 0.98 for globulins, NEFA, and several enzymes that were log-transformed to correct for their lack of normality (Table 2). The statistical model showed a relevant herd variance component for albumin, urea, and glucose, for several hepatic markers (alanine aminotransferase and direct and total bilirubins), and for calcium (Table 3). No significant fixed effect was detected for triglycerides, lactate dehydrogenase, magnesium, and potassium. General confidence intervals were calculated for all of these variables and reported in Table 3. The limited sensitivity of these blood parameters to parity, phase of lactation, and season of production in clinically normal dairy cows is not surprising because, for many of them, variation is limited by homeostatic control systems. Moreover, except for phosphorous, all of the minerals had a coefficient of variation <10%.

A low herd variation (<20% of total variation) was observed for creatinine (16%), AST (17%), ALP (10%), and chlorine (18%). Total protein, globulin, GGT, and CK had a herd variation of 20%, whereas it increased for NEFA (27%), cholesterol (23%), phosphorus (26%), and sodium (24%). For all of these variables, no interaction was observed among main fixed factors and, therefore, specific confidence intervals were calculated when a significant effect of parity (Table 4), stage of lactation (Table 5), and season existed (Table 6). Protein metabolism markers were affected by parity (Table 4). Consistent with the findings of Blum et al. (1983), total protein and globulin concentrations tended to be higher in older cows. The same blood parameters were also higher in summer (Table 6). As albumin was unaffected by season, the seasonal difference observed for total protein and globulin concentration was not a consequence of dehydration. It is more likely that the higher values recorded in the summer for globulin, as well as for creatinine, were a metabolic response of the lactating cow to the hot environment. Heat stress has been shown to increase catabolism of AA for energy (Ronchi et al., 1999; Abeni et al., 2007). Some of these AA could be derived from the protein mobilization of muscle tissue, which would support the summer increase in the plasma levels of creatinine observed in the current study. Increased protein catabolism could also explain the greater summer levels of creatinine through an increased renal activity.

Table 2. Analyzed blood parameters in lactating Holstein cows: number of samples after outlier deletion, mean, median, standard deviation, indexes of skewness and kurtosis, and W value of Shapiro-Wilk test for normality

Parameter[1]	No.	Mean	Median	Skewness	Kurtosis	W	SD
Protein and energy metabolism							
Total protein (g/L)	735	82	81	0.2826	−0.1484	0.99	6.1
Globulin (g/L)	734	45	44	0.6091	0.1656	0.97[1]	6.6
Albumin (g/L)	732	37	37	−0.1701	−0.2713	0.98	2.5
Urea (mmol/L)	738	4.6	4.6	0.1626	−0.0221	0.99	1.2
Creatinine (μmol/L)	736	65	65	0.4187	0.0190	0.98	9.3
Glucose (mmol/L)	736	3.2	3.2	−0.1587	−0.1941	0.99	0.4
NEFA (mEq/L)	721	0.19	0.15	1.6023	2.4285	0.84[2]	0.14
Triglycerides (mmol/L)	734	0.14	0.13	0.3840	−0.0430	0.98	0.04
Enzymes and hepatic markers							
ALT (U/L)	738	32	33	0.0033	−0.2951	0.99	7.4
AST (U/L)	723	83	80	0.9562	1.0290	0.95[2]	16.8
ALP (U/L)	728	107	101	0.8781	0.7788	0.95[2]	37
GGT (U/L)	729	24	23	0.9323	1.1008	0.94[2]	6.3
LDH (U/L)	730	2,146	2,105	0.6237	0.7755	0.97[2]	346
CK (U/L)	730	124	110	2.9293	14.1929	0.77[2]	55
Cholesterol (mmol/L)	737	5.5	5.4	−0.0042	−0.0954	0.99	1.4
Direct bilirubin (μmol/L)	733	1.5	1.3	0.8953	0.4309	0.94[2]	0.5
Total bilirubin (μmol/L)	731	4.9	4.6	0.7935	0.4092	0.95[2]	1.4
Mineral							
Calcium (mmol/L)	733	2.4	2.4	−0.2873	0.2418	0.99	0.1
Phosphorus (mmol/L)	738	1.8	1.5	0.1825	−0.0335	0.99	0.3
Magnesium (mmol/L)	732	0.93	0.87	0.3918	0.6118	0.99	0.09
Sodium (mmol/L)	734	136	134	−0.1331	0.2039	0.99	3
Potassium (mmol/L)	736	4.0	3.7	0.0491	−0.0207	0.99	0.4
Chloride (mmol/L)	729	99	96	0.3332	0.0744	0.99	4.3

[1]ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; GGT = gamma glutamyl transferase; LDH = lactate dehydrogenase; CK = creatinine kinase.

[2]Parameter submitted to logarithmic transformation.

The outcomes of this study (Table 3) confirm that blood glucose is an insensitive marker of energy status in cattle because of its homeostatic regulation (Herdt, 2000; Grünwaldt et al., 2005). The plasma NEFA concentration is more closely related to cow energy status and depot fat mobilization as a consequence of a negative energy balance (Block et al., 2001). In early lactation (Table 5), NEFA concentration reflects the mobilization of lipid reserves to compensate for the imbalance between nutrients consumed by the cow and nutrients secreted in milk (van Knegsel et al., 2007; Grummer, 2008). In mid lactation, NEFA concentrations are relatively low because energy balance becomes positive and cows replete the mobilized tissue reserves

Table 3. Analyzed blood parameters in lactating Holstein cows: mean and confidence interval (>95%) of variables with herd variation ≥30% of total variance or without significant effect of the fixed factors included in the statistical model

Parameter	Herd variation[1]	Mean	CI
Protein and energy metabolism			
Albumin (g/L)	0.35	37	33–41
Triglycerides (mmol/L)	0.14	0.14	0.08–0.20
Urea (mmol/L)	0.56	4.6	2.5–6.7
Glucose (mmol/L)	0.40	3.2	2.6–3.8
Enzymes and hepatic markers			
ALT[2] (U/L)	0.40	32	20–45
LDH[3] (U/L)	0.14	2,095	1,499–2,930
Direct bilirubin (μmol/L)	0.33	1.5	0.8–2.8
Total bilirubin (μmol/L)	0.32	5.1	2.8–9.3
Mineral			
Calcium (mmol/L)	0.38	2.4	2.2–2.6
Magnesium (mmol/L)	0.29	0.93	0.79–1.07
Potassium (mmol/L)	0.20	4.0	3.3–4.6

[1]Percent total variance.

[2]Alanine aminotransferase.

[3]Lactate dehydrogenase (parameter submitted to logarithmic transformation).

Table 4. Analyzed blood parameters in lactating Holstein cows: mean values and confidence intervals (>95%) of variables affected by parity

	Parity			
	Primiparous		Multiparous	
Parameter[1]	Mean	CI	Mean	CI
Protein and energy metabolism				
Total protein (g/L)	80	70–90	83	74–92
Globulin[2] (g/L)	43	33–55	45	33–62
Creatinine (μmol/L)	67	51–83	64	51–78
Enzymes and hepatic markers				
ALP[2] (U/L)	116	66–203	90	46–173
GGT (U/L)	23	13–33	24	16–32
CK[2] (U/L)	122	60–251	103	55–192
Mineral				
Phosphorus (mmol/L)	1.8	1.2–2.4	1.7	1.4–2.1

[1]ALP = alkaline phosphatase; GGT = gamma glutamyl transferase; CK = creatinine kinase.

[2]Parameter submitted to logarithmic transformation.

(Blum et al., 1983; Walters et al., 2002). The higher ALP activity observed in primiparous cows (Table 4) can be associated with the increased osteoblastic activity that occurs in young growing cattle (Meyer and Harvey, 2004). The summer decrease of the same enzyme (Table 6) was an endocrine acclimation response of cattle to the hot environment (Ronchi et al., 1999). Indeed, Abeni et al., (2007) proposed ALP as a plasma marker of heat stress in dairy cattle. Increased serum values of GGT usually reflect liver damage in dairy cattle (Moore, 1997). However, the significant parity effect observed for this parameter in healthy cows (Table 4) could be the result of major productive stress in multiparous than in primiparous cows.

In domestic species, CK is mainly used as a specific marker of skeletal muscle injury (Hoffmann and Solter, 2008). No previous studies have reported on the effect of parity on CK activity of cattle. In horses, CK tends to be higher in younger and untrained animals (Harris et al., 1998). The increase in CK observed in primiparous cows (Table 4) could derive from the physical stress caused by the competitive pressure when they are mixed with more experienced cows (Val-Laillet et al., 2009). Multiparous cows are generally dominant over primiparous ones and frequently show their social dominance through aggressive behaviors addressed toward subordinate cattle (Harris et al., 2007). Blood concentration of several enzymes and hepatic markers was increased by the progress of lactation (Table 5). This trend has been commonly observed for cholesterol (Blum et al., 1983; Herdt and Smith, 1996) and it has been attributed to the changes in serum lipoprotein concentrations during lactation (Raphael et al., 1973). No indications were found in the literature to explain the recorded trends observed for the other parameters that were similarly affected by stage of lactation (AST, GGT, and CK).

Regarding blood minerals, higher phosphorus concentrations in young cows (Table 4) were reported by McAdam and O'Dell (1982). This increase has been

Table 5. Analyzed blood parameters in lactating Holstein cows: mean values and confidence intervals (>95%) of variables affected by stage of lactation

	Stage of lactation			
	Early		Mid	
Parameter[1]	Mean	CI	Mean	CI
Protein and energy metabolism				
NEFA[2] (mEq/L)	0.22	0.06–0.83	0.11	0.03–0.34
Enzymes and hepatic markers				
AST (U/L)	29	19–39	36	25–46
GGT[2] (U/L)	22	14–35	24	15–37
CK[2] (U/L)	107	59–193	111	53–231
Cholesterol (mmol/L)	4.9	2.9–6.9	5.9	4.1–7.7

[1]AST = aspartate aminotransferase; GGT = gamma glutamyl transferase; CK = creatinine kinase.

[2]Parameter submitted to logarithmic transformation.

Table 6. Analyzed blood parameters in lactating Holstein cows: mean values and confidence intervals (>95%) of variables affected by the season of production

	Season of production			
	Summer		Winter	
Parameter	Mean	CI	Mean	CI
Protein and energy metabolism				
Total protein (g/L)	83	72–94	80	73–88
Globulin[1] (g/L)	45	34–61	44	33–59
Creatinine (μmol/L)	68	53–83	63	50–77
Enzymes and hepatic markers				
ALP[1,2] (U/L)	88	46–171	105	55–200
Mineral				
Phosphorus (mmol/L)	1.8	1.4–2.3	1.7	1.3–2.2
Sodium (mmol/L)	135	131–139	137	131–143
Chloride (mmol/L)	98	90–106	100	92–108

[1]Parameter submitted to logarithmic transformation.
[2]ALP = alkaline phosphatase.

related to higher growth hormone activity, promoting intestinal phosphate absorption and renal phosphate re-absorption (Meyer and Harvey, 2004). On the contrary, in the literature, no consistent effect of the season on plasma phosphorous exists (Table 6). Kume et al. (1986) reported its decrease in cows kept in a hot environment, whereas more recently, Calamari et al. (2007) observed an opposite trend. Because phosphorus is not directly involved in the mineral losses caused by the hot environment (NRC, 2001), the increase seen in this study might be related to changes in diet formulation made to boost energy intake and the provision of sodium through Na-phosphates. Along with water, sodium, potassium, and chlorine are, in fact, important constituents of sweat, and sweating is a major thermoregulatory mechanism to dissipate excess body heat when lactating cows are exposed to a hot environment (Kadzere et al., 2002). This acclimation strategy could support the lower mean values for sodium and chlorine recorded in summer samples (Table 6).

The approach adopted in present study for the calculation of confidence intervals, which took into account the effects of parity, stage of lactation, and season of production, will possibly improve the accuracy of the biochemical profile as a tool for the assessment of the metabolic status of lactating Holstein cows. The calculated confidence intervals might be mainly used at herd level to detect alert situations when at least 5% of the sampled cows would fall outside of the calculated reference interval for a given parameter.

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