J. Dairy Sci. 103:572–582
https://doi.org/10.3168/jds.2019-16355

Analysis of milk leukocyte differential measures for use in management practices for decreased mastitis incidence

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ABSTRACT

The aim of this study was to assess the usefulness of measures derived from milk leukocyte differential (MLD) in practices that improve fresh cow mastitis monitoring and decrease mastitis incidence. Quarter milk samples were collected from Holstein and Jersey cows on d 4 and 11 postcalving. Samples were analyzed using MLD, whereby cell counts and quarter infection diagnosis were obtained. Measures derived from MLD included cell scores (total leukocyte, neutrophil, macrophage, and lymphocyte scores), cell proportions (neutrophil, macrophage, and lymphocyte percentages), cell thresholds (total leukocyte, neutrophil, macrophage, and lymphocyte thresholds), and MLD diagnosis at different threshold settings (A, B, and C). Microbiological culturing of milk samples was used to determine infection status to compare the MLD diagnosis and serve as an indicator of infection. Measures derived from the microbiological analysis included occurrence of major pathogens, minor pathogens, and infection. Data analysis was based on a linear mixed model, which was used on all measures for the estimation of the fixed effects of breed, lactation number, day of sample collection, time of sampling, and quarter location, and the random effects of animal and week of sampling. All the fixed effects studied were significant for one or more of the analyzed measures. The results of this study showed that MLD-derived measures justify further study on their use for management practices for mastitis screening and prevention in early lactation.
Key words: mastitis incidence, mastitis management, milk leukocyte differential

INTRODUCTION

Mastitis is well known as a cause of economic loss, due to reduced production, diagnostic and treatment costs, and premature culling of cows (Halasa et al., 2007). The disease includes both clinical mastitis as well as subclinical mastitis. Mastitis has profound effects on many aspects of dairy production because of its association with decreased yields and changes in milk quality and composition (Bobbo et al., 2017). Subclinical mastitis is especially concerning because it often remains undetected due to the lack of external symptoms while the underlying infection and its effects persist. Somatic cell count is one of the most widely used monitoring tools for mastitis (Damm et al., 2017). This measurement quantifies the total number of somatic cells in milk (cells/mL) and is used to evaluate udder health, milk quality, and severity of infection, with cell count levels at or above 100×10^3 cells/mL generally indicating inflammation (Schwarz et al., 2011b; Hand et al., 2012; Bobbo et al., 2017). However, quantification of the different cell types and their proportions is not possible using SCC alone. For this purpose, the milk leukocyte differential (**MLD**) has been developed. Milk leukocyte differential is a tool used to identify and quantify the somatic cells in milk that have roles related to the immune response in the mammary gland. By enabling the monitoring of changes in cell proportions, it can be used to detect inflammation in the early stages of infection before SCC levels become alarming (Pilla et al., 2012, 2013). Research to determine the merit of MLD measures and their possible advantage over SCC measures for diagnosing mastitis has been scarce so far. Therefore, the objective of this study was to evaluate MLD measures for use in management practices that improve subclinical mastitis screening in early lactation.

MATERIALS AND METHODS

Animals

Animal use was approved by the North Carolina State University (**NCSU**) Institutional Animal Care and Use Committee. Quarter milk samples were collected from enrolled recently calved dairy cows on both d 4

Received January 23, 2019.
Accepted September 20, 2019.
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and 11 postcalving (calving date treated as d 0) from December 2016 to November 2017. The study included a total of 127 animals from the Holstein (**HO**, n = 82) and Jersey (**JE**, n = 45) breeds ranging from lactation 1 to 6. Cows were housed at the Dairy Education Unit at NCSU. The herd had an average milk yield of 11,762 kg/yr and an average bulk tank SCC of 177,000 cells/mL during the study. Animals were fed a TMR of corn silage, sorghum silage, grass hay, soy hulls, citrus pulp, and grain mix. Dry cows had free access to pastures or freestalls at their discretion, while the milking herd had limited access to pastures and were milked twice a day at a 12-h interval in a double-10 parallel milking parlor. Cows treated with antibiotics from the period of a month before calving to d 4 postcalving or treated between d 4 and 11 postcalving were not eligible for the study.

Sample Collection and Analysis

Quarter milk samples (n = 987) were collected either during the morning (a.m.) or evening (p.m.) milking in quarter-based sampling chambers (Q4, Advanced Animal Diagnostics, Inc., Morrisville, NC) for determination of MLD, and aseptically collected quarter milk samples (n = 973) were placed in 13-mL vials for microbiological culturing. Due to miscellaneous circumstances, 14 samples destined for microbial culturing were lost; consequently, the number of samples analyzed with MLD is greater than those cultured. Before collection, quarters were aseptically prepared using steps outlined by Barnes-Pallesen et al. (1987). Samples for microbiological culturing were collected from the foremilk after expression of 2 to 3 streams of milk, after which the samples for MLD analysis were collected. Samples were then transported to the NCSU Veterinary College, where samples underwent MLD analysis and duplicate samples were frozen at −20°C for subsequent microbiological analysis.

Microbiological Analysis

In total, 973 sterile milk samples were collected on d 4 (n = 511) and d 11 (n = 462) postcalving. A total of 95 samples were found to be contaminated and are not reported on further. Samples were analyzed within 1 wk of collection. Procedures for bacteriologic culture and identification were consistent with those previously published (Hogan et al., 1999). Milk samples were quickly thawed and shaken for 15 s, and 0.1 mL of milk was plated on trypticase soy agar with 5% sheep blood (BBL TSA II 5% SB agar; Becton, Dickinson and Co., Sparks, MD). Plates were incubated at 36°C and examined after 24 and 48 h of incubation. A culture was classified as contaminated if growth of 3 or more microbial species was observed (Dohoo et al., 2011). Noncontaminated cultures were classified as containing major pathogen species (**MaP**), minor pathogen species (**MiP**), or no growth. The major pathogens cultured included *Staphylococcus aureus*, *Streptococcus* spp., *Trueperella pyogenes*, *Enterococcus* spp., *Pantoea* spp., coliform bacteria (e.g., *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella* spp.), *Serratia marcescens*, and miscellaneous fungal species. Minor pathogens included *Corynebacterium* spp., *Actinomyces* spp., *Acinetobacter* spp., and CNS. Infection classification criteria followed previously published methods (Anderson et al., 2010). Quarters were classified as infected if they contained any MaP at any concentration or MiP at a concentration of ≥100 colonies/mL. Quarters containing both MaP and MiP were classified as containing MaP and counted toward the total number of infected quarters.

Milk Leukocyte Differential

Samples were analyzed using the AAD QScout Farm Lab (Advanced Animal Diagnostics, Inc., Morrisville, NC). Cell counts are reported as total leukocytes, which represent the sum of neutrophils, macrophages, and lymphocytes. The total leukocyte count differs from the SCC in that epithelial cells are not included. The instrument also provides a diagnosis for mastitis for each quarter, which is reported as positive, negative, or borderline. To achieve normality of the data, transformation of cell counts was performed using the same equation used to convert SCC to somatic cell score [SCS = $\log_2$(SCC/100,000) + 3], resulting in scores for each cell type (neutrophils, macrophages, and lymphocytes) and total leukocytes. The percentage of each cell type based on the total leukocyte count was calculated, and neutrophil, macrophage, and lymphocyte percentages were obtained.

The AAD QScout Farm Lab was evaluated by comparing the diagnosis given by the machine with the infection status according to microbiological culturing. Samples were analyzed using the early lactation setting of the AAD QScout Farm Lab, which includes 18 diagnostic settings (1–18). Estimates of sensitivity (**Se**), specificity (**Sp**), and accuracy (**Ac**) were calculated for all settings, and 3 threshold diagnostic settings were chosen for further analysis based on the default factory setting (setting 12, **diagA)**, highest Sp (setting 18, **diagB)**, and highest Se (setting 2, **diagC**). Cell thresholds that best represented infection status were obtained according to published methods (Youden, 1950), with a receiver operating characteristic curve

being used to plot the true-positive rate (Se) and false-positive rate (1 − Sp) of different cell thresholds for every cell type (total leukocytes, neutrophils, macrophages, and lymphocytes).

Statistical Analyses

To estimate the effects of breed, lactation, day of sample collection, time of sampling, quarter location, animal, and week of sampling (week within sampling period) on cell score (total leukocyte, neutrophil, macrophage, and lymphocyte scores), we used cell percentage (neutrophil, macrophage, and lymphocyte percentages), MLD diagnosis at different settings (diagA, diagB, diagC), pathogen type occurrence (MaP, MiP), infection, and infection according to cell thresholds in the following model:

$$y_{ijklmno} = \mu + Breed_i + Lactation_j + Day_k + TOD_l + Quarter_m + ID_{n(i)} + Week_o + e_{ijklmno},$$

where $y_{ijklmno}$ is the investigated measure; μ is the overall mean; $Breed_i$ is the fixed effect of the ith class of breed (i = HO, JE); $Lactation_j$ is the fixed effect of the jth class of lactation number (j = 1, ≥2); Day_k is the fixed effect of the kth class of day of sample collection (k = 4, 11); TOD_l is the fixed effect of the lth class of time of day (l = a.m., p.m.); $Quarter_m$ is the fixed effect of the mth class of quarter location [m = left rear (**LR**), right rear (**RR**), left front (**LF**), right front (**RF**)]; $ID_{n(i)}$ is the random effect of the nth class of animal within the ith class of breed; $Week_o$ is the random effect of the oth class of week of sampling (week within sampling period, 1–48 wk); and $e_{ijklmno}$ is the random residual. Vectors for $ID_{n(i)}$, $Week_o$, and $e_{ijklmno}$ were assumed normally and independently distributed with mean equal to 0 and variance equal to the estimated variances σ_c^2, σ_w^2, and σ_e^2, respectively. The model was fitted using PROC GLIMMIX in SAS (version 9.4, SAS Institute Inc., Cary, NC). *F*-values and *P*-values for all effects, as well as least squares means estimates, were determined using the same procedure, whereby categorical measures were transformed from the underlying liability scale to probability scale using previously published methods (Zwald et al., 2006). Statistical significance for all effects was considered present at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Microbiological Analysis

Culture classification and organism prevalence results for the remaining samples after removal of contaminated samples are reported in Table 1. After incubation of the samples, 197 (22.44%) cultures had growth of 1 or more microbial species, while 681 (77.56%) showed no growth. The proportion of cultures showing positive growth compared with negative growth is similar to that reported by Godden et al. (2017) for early lactation but lower than values found in other studies (Jashari et al., 2016; Gonçalves et al., 2017). Differences between the present study and other studies for the percentage of growth-positive cultures may be due to many factors, such as differences in sampling strategy, total quarters sampled, stage of lactation of sampled animals, criteria for growth classification, volume of milk that was plated (0.1 mL), and herd-specific risk factors. Of those samples that resulted in growth, 110 (55.84%) were classified as containing MaP. *Staphylococcus aureus* was the most prevalent MaP, followed by *Streptococcus dysgalactiae*. The percentage of *Staph. aureus* (23.86%) and *Staph. aureus* dual infections (2.54%) found was higher than seen in many well-managed dairy herds, and this finding should be considered in assessing the results. The remaining MaP were less prevalent and included *Streptococcus uberis*, *Klebsiella* spp., *Escherichia coli*, *Enterobacter aerogenes*, *Enterococcus* spp., *Trueperella pyogenes*, *Pantoea* spp., and *Serratia marcescens*. Minor pathogens were present in 87 (44.16%) of growth-positive cultures, with CNS being the most prevalent MiP found, followed by *Corynebacterium* spp. Other MiP found were *Actinomyces* spp. and *Acinetobacter* spp. Gonçalves et al. (2017) observed similar results for species of microorganisms isolated, frequency of specific pathogens, and proportion of MaP to MiP. Based upon our definition of infection, a total of 167 quarters (19.02%) were classified as infected, while 711 (80.98%) were classified as not infected. Of those samples that were classified as infected, 108 samples (64.67%) were from animals at d 4 of sample collection and 59 samples (35.33%) were from animals at d 11 of sample collection. Eighty-nine of the infected samples (53.29%) were from animals in their first lactation, which represents 35% of samples collected, and 78 samples (46.71%) were from animals with 2 or more lactations, which represents 65% of the samples collected. The comparability of our infection classification results with other studies is complicated by the lack of a widespread consensus on the pathogenicity of certain microorganisms, sampling and culturing methodologies, and culture classification in mastitis research. Our classification scheme is one of several that could be used.

Milk Leukocyte Differential

Descriptive statistics for cell counts, cell scores, cell percentages, cell thresholds, and MLD diagnosis

Table 1. Milk culture classification and organism prevalence

Classification	No. of cultures	%[1]	%[2]	Mean count (cells/mL)			
				Total leukocytes	Neutrophils	Macrophages	Lymphocytes
No growth	681	77.56		129.80×10^3	70.98×10^3	29.53×10^3	29.58×10^3
Major pathogens	110	12.53	55.84	$1{,}406.00 \times 10^3$	988.30×10^3	159.30×10^3	257.7×10^3
Staphylococcus aureus	47	5.35	23.86				
Staphylococcus aureus dual infections[3]	5	0.57	2.54				
Streptococcus dysgalactiae	26	2.96	13.20				
Streptococcus dysgalactiae dual infections[4]	7	0.80	3.55				
Streptococcus uberis	3	0.34	1.52				
Escherichia coli + *Enterococcus* spp.	1	0.11	0.51				
Enterobacter aerogenes	1	0.11	0.51				
Enterococcus spp.	1	0.11	0.51				
Pantoea spp.	1	0.11	0.51				
Serratia marcescens	2	0.23	1.02				
Trueperella pyogenes + *Corynebacterium* spp.	1	0.11	0.51				
Enterococcus spp. + CNS	1	0.11	0.51				
Fungi	14	1.59	7.11				
Minor pathogens	87	9.91	44.16	201.70×10^3	105.40×10^3	47.56×10^3	38.05×10^3
Actinomyces spp.	1	0.11	0.51				
CNS	55	6.26	27.92				
CNS dual infections[5]	4	0.46	2.03				
Corynebacterium spp.	27	3.08	13.71				
Total	878	100.00	100.00				

[1]Percentage calculated on the total amount of noncontaminated cultures (n = 878).

[2]Percentage calculated on the total amount of growth positive cultures (n = 197).

[3]*Staphylococcus aureus* in combination with *Escherichia coli* (1), *Trueperella pyogenes* (1), *Corynebacterium* spp. (1), and *Streptococcus dysgalactiae* (2).

[4]*Streptococcus dysgalactiae* in combination with *Escherichia coli* (1), *Serratia marcescens* (1), *Klebsiella* spp. (1), and CNS (4).

[5]CNS in combination with *Acinetobacter* spp. (2) and *Corynebacterium* spp. (2).

thresholds are given in Table 2. Milk leukocyte differential diagnosis was obtained for all 987 quarter milk samples, but records for 111 samples were not kept due to incomplete information; these samples were classified as unknown and excluded from diagnosis analyses. The results for the evaluation of the AAD QScout Farm Lab, using the microbiological culturing infection status as the gold standard, were as follows: the first threshold setting (diagA) had 44.72% Se, 92.69% Sp, and 84.55% Ac; the second threshold setting (diagB) had 34.93% Se, 95.40% Sp, and 84.63% Ac; and the third threshold setting (diagC) had 60.27% Se, 83.60% Sp, and 79.46% Ac. Our estimates are higher for Se and similar for Sp compared with those obtained by Godden et al. (2017) (Se = 12.7 to 39.1, Sp = 82.1 to 95.2), who performed a similar study evaluating the MLD test using the same machine and duplicate sampling for early-lactation animals. Based on the values for Se and Sp of the 3 settings, the "best" threshold depends on whether the objective is to decrease the number of quarters that are treated without having a true IMI (false positives) or the number of quarters that are not treated while being infected (false negatives). This investigation is the first published study to obtain cell thresholds that maximize both Se and Sp and to evaluate their use in mastitis detection. The values for cell concentration that maximize the Se and Sp, and therefore best represent the difference between a positive or negative diagnosis, were obtained by calculating the Youden's index (Youden, 1950) for various thresholds of every cell type. The selected cell concentration threshold was 162×10^3 cells/mL (Se = 61.00%, Sp = 80.03%) for total leukocyte count, 93×10^3 cells/mL (Se = 59.14%, Sp = 83.48%) for neutrophils, 56×10^3 cells/mL (Se = 47.56%, Sp = 89.06%) for macrophages, and 47×10^3 cells/mL (Se = 56.70%, Sp = 84.08%) for lymphocytes. Estimates of Se and Sp among cell thresholds are very similar, with total leukocyte threshold having the highest Se (61.00%) and macrophage threshold the highest Sp (89.06%). The receiver operating characteristic curve for each cell type threshold is illustrated in Figure 1. Cell concentration thresholds were also calculated for each breed, with thresholds for JE being 207×10^3 cells/mL (Se = 71%, Sp = 85%) for total leukocytes, 132×10^3 cells/mL (Se = 69%, Sp = 90%) for neutrophils, 47×10^3 cells/mL (Se = 67%, Sp = 86%) for macrophages, and 47×10^3 cells/mL (Se = 72%, Sp = 85%) for lymphocytes. For HO, the thresholds were 105×10^3 cells/mL (Se = 65%, Sp = 73%) for total leukocytes, 47×10^3 cells/mL (Se = 70%, Sp = 70%) for neutrophils, 42×10^3 cells/mL (Se = 41%, Sp = 87%) for macrophages, and 22×10^3 cells/mL (Se = 67%, Sp = 68%) for lymphocytes. Further analysis with breed-specific thresholds was not done due to the lack of a balanced number of animals from each breed group. Thresholds within this article were obtained using data available for the current analysis. Further research should consider the effects of sample size, different herds, and environmental conditions to assess threshold robustness.

In the present study, we did not obtain SCC readings from the milk at sampling for comparison with MLD cell measures, particularly total leukocyte count, and total leukocyte score. However, D. Nolan and J. H. C. Costa (University of Kentucky, Lexington, personal communication) have recently found that SCC measured with SomaCount FC (Bentley Instruments Inc., Chaska, MN) and total leukocyte count measured with MLD share a high Pearson correlation. Similar results were found when both measurements were transformed to cell scores. These high correlations may be due to the low proportion of epithelial cells, which are included in SCC measurements and not in MLD, compared with other cells in bovine milk (Boutinaud and Jammes, 2002; Schwarz et al., 2011a).

Estimation of F-Values, Significance, and Least Squares Means

F-value and *P*-value results as well as least squares means estimates are presented in Tables 3 and 4, respectively. The effect of breed was significant for all cell score measures considered and for all cell threshold measures except lymphocyte threshold. Jersey animals had on average higher cell scores and cell threshold infection rates; total leukocyte score (JE = 3.24, HO = 2.68, $P = 0.012$), neutrophil score (JE = 2.23, HO = 1.64, $P = 0.015$), macrophage score (JE = 1.06, HO = 0.64, $P = 0.033$), lymphocyte score (JE = 1.02, HO = 0.42, $P = 0.009$), total leukocyte threshold (JE = 0.28, HO = 0.15, $P = 0.020$), neutrophil threshold (JE = 0.25, HO = 0.13, $P = 0.025$), and macrophage threshold (JE = 0.16, HO = 0.08, $P = 0.020$). One possible explanation for this phenomenon is the difference in milk volume produced by these 2 breeds, which would cause a higher concentration of somatic cells in Jerseys. Breed has previously been found to be a significant factor for milk yield, milk and protein composition measures, and milk coagulation properties (Bobbo et al., 2017; Stocco et al., 2017). Several studies (Abebe et al., 2016; Hiitiö et al., 2017) have found differences between breeds with regard to health and disease resistance. Abebe et al. (2016) found that the presence of mastitis was significantly higher in Holstein × Zebu crosses than in purebred Zebu cattle, while Hiitiö et al. (2017) reported significantly lower SCC and occurrence

Table 2. Descriptive statistics for milk leukocyte differential (MLD) and diagnosis measures by day of sample collection

	Mean		Median		Minimum value		Maximum value		SD	
Measure	Day 4	Day 11	Day 4	Day 11	Day 4	Day 11	Day 4	Day 11	Day 4	Day 11
Cell counts[1]										
Total leukocytes	368.80	227.18	104.00	59.50	15.00	7.00	12,149.00	17,250.00	1,153.88	1,019.03
Neutrophils	235.73	144.24	50.00	28.00	5.00	3.00	9,040.00	12,750.00	851.75	747.12
Macrophages	60.78	33.18	27.00	16.00	2.00	0.00	1,614.00	984.00	135.00	72.32
Lymphocytes	72.43	49.71	24.00	12.00	2.00	1.00	2,152.00	3,517.00	189.19	211.49
Cell scores										
Total leukocytes	3.35	2.53	3.06	2.25	0.26	−0.64	9.92	10.43	1.69	1.66
Neutrophils	2.37	1.50	2.00	1.16	−1.32	−2.05	9.50	9.99	1.83	1.83
Macrophages	1.18	0.44	1.11	0.36	−2.64	−10.29	7.01	6.30	1.59	1.50
Lymphocytes	1.17	0.23	0.94	−0.06	−2.64	−3.64	7.43	8.13	1.72	1.85
Cell percentages										
Neutrophils	51.95	50.82	51.92	50.65	9.63	13.79	77.88	89.09	10.43	11.98
Macrophages	25.13	27.56	23.75	25.81	4.63	0.00	74.13	83.33	12.00	14.70
Lymphocytes	24.11	22.02	22.74	21.45	0.40	2.22	84.18	63.08	8.24	8.45
Cell thresholds[2]										
Total leukocytes	0.34	0.18								
Neutrophils	0.30	0.17								
Macrophages	0.22	0.11								
Lymphocytes	0.27	0.13								
MLD diagnosis[2,3]										
DiagA	0.15	0.11								
DiagB	0.10	0.07								
DiagC	0.27	0.16								

[1]Unit for cell count is $\times 10^3$ cells/mL.

[2]Occurrence of infection according to threshold.

[3]DiagA = positive diagnosis threshold setting A; DiagB = positive diagnosis threshold setting B; DiagC = positive diagnosis threshold setting C.

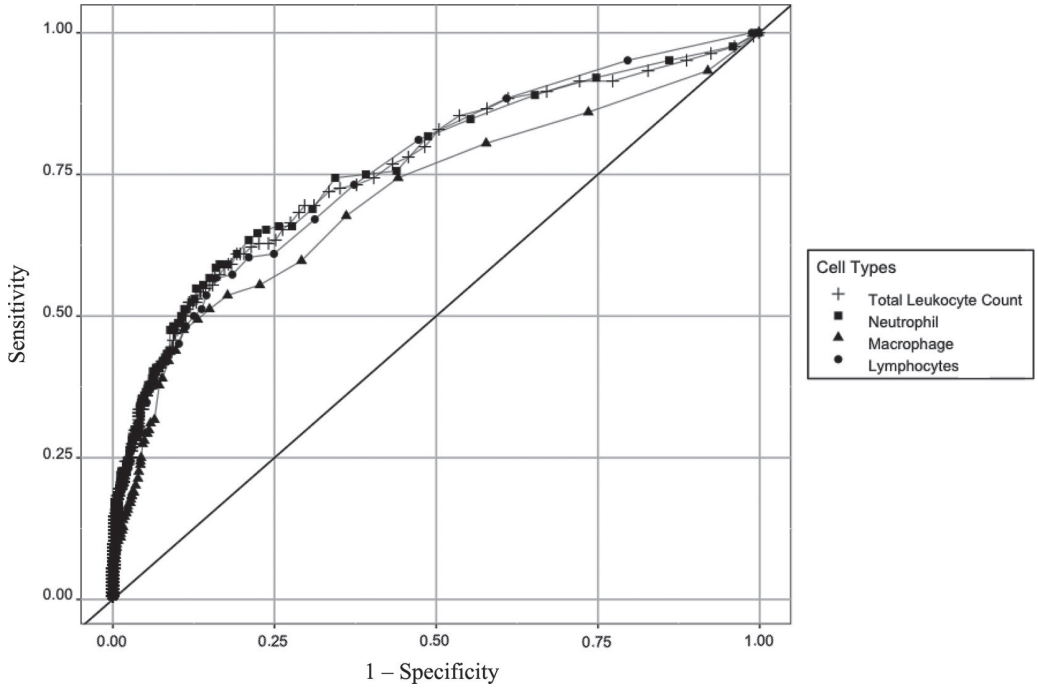

Figure 1. Combined receiver operating characteristic curves for cell concentration thresholds.

of subclinical mastitis and chronic subclinical mastitis in the Ayrshire breed than in Holsteins.

The effect of lactation was significant for MaP occurrence and infection. Similar to our study, Pilla et al. (2012) found that lactation number was not significant for any of the cell percentages studied (neutrophils, macrophages, or lymphocytes). Animals in their first lactation had a higher MaP occurrence (1 = 0.14, ≥2 = 0.03, $P = 0.0002$) and infection (1 = 0.26, ≥2 = 0.11, $P = 0.0007$) compared with animals with multiple lactations. These results accord with published findings. Compton et al. (2007) found that the risk of clinical mastitis in heifers is 3 times as high as that in older cows in the early stages of lactation, Barkema et al. (1998) found that the incidence rate of clinical mastitis was higher in heifers than in cows in the first 2 wk of lactation, and Oliveira et al. (2015) reported that multiparous cows had a lower probability of developing CNS infection as compared with primiparous animals. Conversely, other groups have found an opposite effect of lactation number regarding infection. Hiitiö et al. (2017) analyzed 2 decades worth of Finnish health monitoring and milk recording data and reported that the risk of subclinical mastitis and chronic subclinical mastitis increases with increasing parity (i.e., lactation number).

The effect of day of sample collection was significant for all measures considered in this study with the exception of neutrophil percentage, MiP, and a positive diagnosis using diagB. On d 4 of sample collection, animals had on average higher cell scores, lymphocyte percentage, cell threshold infection rate, MaP, infection, and rate of positive diagnosis at diagA and diagC, than animals on d 11 of sample collection. For d 4 and 11, respectively, total leukocyte score was 3.36 and 2.56 ($P < 0.0001$); neutrophil score, 2.35 and 1.51 ($P < 0.0001$); macrophage score, 1.21 and 0.49 ($P < 0.0001$); lymphocyte score, 1.19 and 0.26 ($P < 0.0001$); lymphocyte percentage, 0.23 and 0.22 ($P = 0.0150$); total leukocyte threshold, 0.30 and 0.14 ($P < 0.0001$); neutrophil threshold, 0.25 and 0.13 ($P < 0.0001$); macrophage threshold, 0.16 and 0.08 ($P = 0.0034$); lymphocyte threshold, 0.24 and 0.12 ($P < 0.0001$); MaP, 0.09 and 0.05 ($P = 0.0256$); infection, 0.21 and 0.14 ($P = 0.0095$); diagA, 0.09 and 0.06 ($P = 0.0498$); and diagC, 0.25 and 0.12 ($P = 0.0022$). These results for cell score, cell threshold, and diagnosis measures are expected because the level of somatic cells, specifically neutrophils, is known to be high at parturition and to decline gradually during the first weeks of lactation, even for noninfected animals (Dohoo, 1993). The elevated occurrence of MaP and infection might be due to the

persistence of dry period infections, an increased risk of infection in fresh animals by opportunistic pathogens, or both factors. On d 11, animals had higher macrophage percentage (0.28) than those on d 4 (0.25; $P = 0.0017$). The changes in cell proportions as they relate to SCC increase have been heavily investigated. Pilla et al. (2012) found that macrophage percentage was lower in early lactation and day of sample collection had a significant effect on the percentage. Meanwhile, Damm et al. (2017) found a decreasing trend in macrophage percentage as SCC increased. Therefore, our results may be due to an increase in macrophage percentage as a factor of the previously mentioned decline of neutrophils and lymphocytes after the first days of lactation.

The effect of time of sampling was significant for neutrophil score, neutrophil percentage, and macrophage percentage. Samples taken in the morning resulted in a higher macrophage percentage (a.m. = 0.28, p.m. = 0.25, $P = 0.0130$), while sampling in the evening resulted in higher neutrophil score (a.m. = 1.73, p.m. = 2.13, $P = 0.0315$) and neutrophil percentage (a.m. = 0.49, p.m. = 0.52, $P = 0.0026$). These results reflect changes in cell proportion that occur during the day and are consistent with the observed diurnal variation of cells by Olde Riekerink et al. (2007), specifically changes in proportions of neutrophils and macrophages post milking. Finally, the effect of quarter position was significant for macrophage percentage, lymphocyte percentage, MiP, and infection. For these measures, at least one quarter had an estimate that differed significantly from 1 or more quarters; macrophage percentage (LF = 0.29, LR = 0.25, RF = 0.27, RR = 0.25, $P = 0.0009$), lymphocyte percentage (LF = 0.21, LR = 0.24, RF = 0.22, RR = 0.24, $P < 0.0001$), MiP (LF = 0.08, LR = 0.10, RF = 0.05, RR = 0.17, $P = 0.0030$), and infection (LF = 0.15, LR = 0.17, RF = 0.14, RR = 0.24, $P = 0.0419$). Quarter position has been found in previous studies to not be significantly associated with cell percentages (Pilla et al., 2012, 2013). Findings on the effect of quarter position have been mixed (Hammer et al., 2012; Abebe et al., 2016). While Abebe et al. (2016) found a significantly higher proportion of hind quarters with a positive California Mastitis tTest result compared with fore quarters, Hammer et al. (2012) reported no significant association between quarter position and mastitis occurrence. Although our results indicate an effect of quarter location for some of the measures studied, the small magnitude of the differences between quarters and the lack of apparent biological significance leaves this finding inconclusive. Furthermore, the implications of a quarter location effect for mastitis research and possible biological explanations are yet to be elucidated. Further studies on quarter susceptibility are warranted.

Table 3. Results of ANOVA

	Fixed effect				
Measure	Breed	Day of sample collection	Lactation	Time of sampling	Quarter
Cell score					
Total leukocytes	6.47**	79.5***	0.8	2.86	0.68
Neutrophils	6.09**	76.53***	0.64	4.64*	0.75
Macrophages	4.64*	71.93***	0.34	0.14	0.61
Lymphocytes	7.06**	90.03***	0.91	2.49	2.53
Cell percentages					
Neutrophils	1.41	2.88	0	9.10**	0.72
Macrophages	1.37	9.95**	0.48	6.20*	5.56***
Lymphocytes	0.09	5.95*	0.21	0.07	11.39***
Cell thresholds					
Total leukocytes	5.54*	25.6***	1.8	0.19	1.0
Neutrophils	5.16*	16.4***	2.01	0.56	1.41
Macrophages	5.41*	8.66**	0.43	0.71	0.48
Lymphocytes	3.8	19.17***	1.28	0.02	2.1
Microbial culturing					
Major pathogen	1.78	5.0*	13.87***	0.51	0.23
Minor pathogen	0.07	0.8	1.39	1.97	4.69**
Infection	0.67	6.75**	11.71***	0	2.75*
MLD diagnosis[1]					
DiagA	1.95	3.86*	0.86	2.31	0.38
DiagB	2.98	2.30	0.27	0.51	0.15
DiagC	2.16	18.28***	0.27	0.42	1.37

[1]MLD = milk leukocyte differential; DiagA = positive diagnosis threshold setting A; DiagB = positive diagnosis threshold setting B; DiagC = positive diagnosis threshold setting C.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 4. Least square means results (SE within parentheses) for the effects of breed, lactation, day of sample collection, time of sampling, and quarter

	Breed		Lactation		Day of sample collection		Time of sampling		Quarter			
Measure	Holstein	Jersey	1	≥2	4	11	a.m.	p.m.	Left front	Left rear	Right front	Right rear
Cell score												
Total leukocytes	2.68	3.24	3.06	2.86	3.36	2.56	2.81	3.11	2.86	2.97	3.01	3.01
	(0.15)	(0.19)	(0.19)	(0.15)	(0.14)	(0.14)	(0.18)	(0.12)	(0.15)	(0.15)	(0.15)	(0.15)
Neutrophils	1.64	2.23	2.03	1.84	2.35	1.51	1.73	2.13	1.82	1.93	1.98	2.00
	(0.16)	(0.20)	(0.20)	(0.16)	(0.15)	(0.15)	(0.20)	(0.13)	(0.16)	(0.16)	(0.16)	(0.16)
Macrophages	0.64	1.06	0.91	0.79	1.21	0.49	0.82	0.88	0.88	0.78	0.92	0.82
	(0.14)	(0.17)	(0.17)	(0.14)	(0.13)	(0.13)	(0.17)	(0.12)	(0.14)	(0.14)	(0.14)	(0.14)
Lymphocytes	0.42	1.02	0.83	0.62	1.19	0.26	0.57	0.87	0.53	0.80	0.71	0.86
	(0.16)	(0.20)	(0.20)	(0.16)	(0.14)	(0.15)	(0.20)	(0.13)	(0.16)	(0.16)	(0.16)	(0.16)
Cell percentages												
Neutrophils	0.50	0.51	0.51	0.51	0.51	0.50	0.49	0.52	0.50	0.50	0.51	0.51
	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
Macrophages	0.27	0.26	0.26	0.27	0.25	0.28	0.28	0.25	0.29	0.25	0.27	0.25
	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
Lymphocytes	0.23	0.23	0.23	0.22	0.23	0.22	0.23	0.23	0.21	0.24	0.22	0.24
	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
Cell thresholds												
Total leukocytes	0.15	0.28	0.25	0.17	0.3	0.14	0.2	0.22	0.17	0.21	0.23	0.23
	(0.03)	(0.06)	(0.05)	(0.04)	(0.04)	(0.03)	(0.05)	(0.03)	(0.04)	(0.04)	(0.05)	(0.05)
Neutrophils	0.13	0.25	0.22	0.15	0.25	0.13	0.17	0.20	0.14	0.19	0.21	0.21
	(0.03)	(0.05)	(0.05)	(0.03)	(0.04)	(0.03)	(0.05)	(0.03)	(0.03)	(0.04)	(0.04)	(0.04)
Macrophages	0.08	0.16	0.13	0.10	0.16	0.08	0.1	0.13	0.10	0.13	0.10	0.13
	(0.02)	(0.04)	(0.04)	(0.03)	(0.03)	(0.02)	(0.03)	(0.03)	(0.03)	(0.03)	(0.03)	(0.03)
Lymphocytes	0.13	0.22	0.20	0.14	0.24	0.12	0.17	0.18	0.12	0.18	0.19	0.21
	(0.03)	(0.05)	(0.04)	(0.03)	(0.03)	(0.03)	(0.04)	(0.03)	(0.03)	(0.04)	(0.04)	(0.04)
Microbial culturing[1]												
Major pathogen	0.05	0.09	0.14	0.03	0.09	0.05	0.06	0.08	0.07	0.06	0.06	0.08
	(0.02)	(0.03)	(0.04)	(0.01)	(0.02)	(0.02)	(0.03)	(0.02)	(0.02)	(0.02)	(0.02)	(0.03)
Minor pathogen	0.1	0.09	0.11	0.08	0.09	0.11	0.12	0.08	0.08	0.10	0.05	0.17
	(0.02)	(0.02)	(0.03)	(0.02)	(0.02)	(0.02)	(0.03)	(0.01)	(0.02)	(0.03)	(0.02)	(0.03)
Infection	0.15	0.19	0.26	0.11	0.21	0.14	0.17	0.17	0.15	0.17	0.14	0.24
	(0.03)	(0.04)	(0.05)	(0.02)	(0.03)	(0.03)	(0.04)	(0.03)	(0.03)	(0.04)	(0.03)	(0.04)
MLD diagnosis[1]												
DiagA	0.06	0.09	0.09	0.06	0.09	0.06	0.05	0.10	0.06	0.08	0.08	0.07
	(0.02)	(0.03)	(0.03)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)	(0.02)
DiagB	0.05	0.09	0.08	0.06	0.08	0.06	0.06	0.08	0.06	0.07	0.08	0.07
	(0.02)	(0.03)	(0.03)	(0.02)	(0.02)	(0.02)	(0.03)	(0.02)	(0.02)	(0.02)	(0.03)	(0.02)
DiagC	0.14	0.22	0.19	0.16	0.25	0.12	0.16	0.19	0.14	0.17	0.21	0.21
	(0.04)	(0.06)	(0.06)	(0.04)	(0.05)	(0.03)	(0.05)	(0.04)	(0.04)	(0.05)	(0.05)	(0.05)

[1]MLD = milk leukocyte differential; DiagA = positive diagnosis threshold setting A; DiagB = positive diagnosis threshold setting B; DiagC = positive diagnosis threshold setting C.

CONCLUSIONS

Milk leukocyte differential is a recently developed tool for diagnosing mastitis by using the changes in somatic cell populations and their overall proportions. This method has been found to have high specificity, correctly diagnosing quarters that are not infected, but it only has moderate sensitivity, as shown by our results and those of previously published studies. We found that for the measures derived from MLD, significant differences exist at the quarter level with regard to breed, lactation, day of sample collection, time of sampling, and quarter position. These results will aid in understanding the differences in somatic cell recruitment as it relates to temporal, spatial, and animal variables. Further research must be done to validate the results of our study in different populations and with pathogen-specific infection before such implementations are possible.

ACKNOWLEDGMENTS

The authors acknowledge the contribution of Dereck Nolan and Joao H. C. Costa from the University of Kentucky for their willingness to share preliminary results of their study about on-farm use of milk leukocyte differential and comparisons with traditional milk quality parameters. We also extend our gratitude to Lake Wheeler Dairy Education Unit staff Gary Cartwright, Michael Veach, and Devan Schreiber for all their help during data collection, and to Keena Mullen for her help in the design of this study.

REFERENCES

Abebe, R., H. Hatiya, M. Abera, B. Megersa, and K. Asmare. 2016. Bovine mastitis: Prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. BMC Vet. Res. 12:270. https://doi.org/10.1186/s12917-016-0905-3.

Anderson, K. L., M. T. Correa, A. Allen, and R. R. Rodriguez. 2010. Fresh cow mastitis monitoring on day 3 postpartum and its relationship to subsequent milk production. J. Dairy Sci. 93:5673–5683. https://doi.org/10.3168/jds.2009-2885.

Barkema, H. W., Y. H. Schukken, T. J. G. M. Lam, M. L. Beiboer, H. Wilmink, G. Benedictus, and A. Brand. 1998. Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts. J. Dairy Sci. 81:411–419. https://doi.org/10.3168/jds.S0022-0302(98)75591-2.

Barnes-Pallesen, F. D., P. Blackmer, A. Britten, R. B. Bushnell, D. M. Van Damme, and F. Welcome. 1987. Laboratory and Field Handbook on Bovine Mastitis. National Mastitis Council, Arlington, VA.

Bobbo, T., P. L. Ruegg, G. Stocco, E. Fiore, M. Gianesella, M. Morgante, D. Pasotto, G. Bittante, and A. Cecchinato. 2017. Associations between pathogen-specific cases of subclinical mastitis and milk yield, quality, protein composition, and cheese-making traits in dairy cows. J. Dairy Sci. 100:4868–4883. https://doi.org/10.3168/jds.2016-12353.

Boutinaud, M., and H. Jammes. 2002. Potential uses of milk epithelial cells: A review. Reprod. Nutr. Dev. 42:133–147. https://doi.org/10.1051/rnd:2002013.

Compton, C. W. R., C. Heuer, K. Parker, and S. McDougall. 2007. Epidemiology of mastitis in pasture-grazed peripartum dairy heifers and its effects on productivity. J. Dairy Sci. 90:4157–4170. https://doi.org/10.3168/jds.2006-880.

Damm, M., C. Holm, M. Blaabjerg, M. N. Bro, and D. Schwarz. 2017. Differential somatic cell count—A novel method for routine mastitis screening in the frame of Dairy Herd Improvement testing programs. J. Dairy Sci. 100:4926–4940. https://doi.org/10.3168/jds.2016-12409.

Dohoo, I. R. 1993. An evaluation of the validity of individual cow somatic cell counts from cows in early lactation. Prev. Vet. Med. 16:103–110. https://doi.org/10.1016/0167-5877(93)90080-D.

Dohoo, I.R., J. Smith, S. Andersen, D.F. Kelton, and S. Godden. Mastitis Research Workers' Conference. 2011. Diagnosing intramammary infections: Evaluation of definitions based on a single milk sample. J. Dairy Sci. 94:250–261. https://doi.org/10.3168/jds.2010-3559.

Godden, S. M., E. Royster, J. Timmerman, P. Rapnicki, and H. Green. 2017. Evaluation of an automated milk leukocyte differential test and the California Mastitis Test for detecting intramammary infection in early- and late-lactation quarters and cows. J. Dairy Sci. 100:6527–6544. https://doi.org/10.3168/jds.2017-12548.

Gonçalves, J. L., R. L. Lyman, M. Hockett, R. Rodriguez, M. V. dos Santos, and K. L. Anderson. 2017. Using milk leukocyte differentials for diagnosis of subclinical bovine mastitis. J. Dairy Res. 84:309–317. https://doi.org/10.1017/S0022029917000267.

Halasa, T., K. Huijpsl, O. Østerås, and H. Hogeveen. 2007. Economic effects of bovine mastitis and mastitis management: A review. Vet. Q. 29:18–31.

Hammer, J. F., J. Morton, and K. Kerrisk. 2012. Quarter-milking-, quarter-, udder- and lactation-level risk factors and indicators for clinical mastitis during lactation in pasture-fed dairy cows managed in an automatic milking system. Aust. Vet. J. 90:167–174. https://doi.org/10.1111/j.1751-0813.2012.00917.x.

Hand, K. J., A. Godkin, and D. F. Kelton. 2012. Milk production and somatic cell counts: A cow-level analysis. J. Dairy Sci. 95:1358–1362. https://doi.org/10.3168/jds.2011-4927.

Hiitiö, H., J. Vakkamäki, H. Simojoki, T. Autio, J. Junnila, S. Pelkonen, and S. Pyörälä. 2017. Prevalence of subclinical mastitis in Finnish dairy cows: Changes during recent decades and impact of cow and herd factors. Acta Vet. Scand. 59:22. https://doi.org/10.1186/s13028-017-0288-x.

Hogan, J. S., R. N. Gonzales, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith. 1999. Laboratory Handbook on Bovine Mastitis. National Mastitis Council, Madison, WI.

Jashari, R., S. Piepers, and S. De Vliegher. 2016. Evaluation of the composite milk somatic cell count as a predictor of intramammary infection in dairy cattle. J. Dairy Sci. 99:9271–9286. https://doi.org/10.3168/jds.2015-10753.

Olde Riekerink, R. G. M., H. W. Barkema, W. Veenstra, F. E. Berg, H. Stryhn, and R. N. Zadoks. 2007. Somatic cell count during and between milkings. J. Dairy Sci. 90:3733–3741. https://doi.org/10.3168/jds.2007-0001.

Oliveira, C. S. F., H. Hogeveen, A. M. Botelho, P. V. Maia, S. G. Coelho, and J. P. A. Haddad. 2015. Cow-specific risk factors for clinical mastitis in Brazilian dairy cattle. Prev. Vet. Med. 121:297–305. https://doi.org/10.1016/j.prevetmed.2015.08.001.

Pilla, R., M. Malvisi, G. G. M. Snel, D. Schwarz, S. König, C. P. Czerny, and R. Piccinini. 2013. Differential cell count as an alternative method to diagnose dairy cow mastitis. J. Dairy Sci. 96:1653–1660. https://doi.org/10.3168/jds.2012-6298.

Pilla, R., D. Schwarz, S. König, and R. Piccinini. 2012. Microscopic differential cell counting to identify inflammatory reactions in dairy cow quarter milk samples. J. Dairy Sci. 95:4410–4420. https://doi.org/10.3168/jds.2012-5331.

Schwarz, D., U. S. Diesterbeck, S. König, K. Brügemann, K. Schlez, M. Zschöck, W. Wolter, and C.-P. Czerny. 2011a. Microscopic dif-

ferential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. J. Dairy Res. 78:448–455. https://doi.org/10.1017/S0022029911000574.

Schwarz, D., U. S. Diesterbeck, S. König, K. Brügemann, K. Schlez, M. Zschöck, W. Wolter, and C. P. Czerny. 2011b. Flow cytometric differential cell counts in milk for the evaluation of inflammatory reactions in clinically healthy and subclinically infected bovine mammary glands. J. Dairy Sci. 94:5033–5044. https://doi.org/10.3168/jds.2011-4348.

Stocco, G., C. Cipolat-Gotet, T. Bobbo, A. Cecchinato, and G. Bittante. 2017. Breed of cow and herd productivity affect milk composition and modeling of coagulation, curd firming, and syneresis. J. Dairy Sci. 100:129–145. https://doi.org/10.3168/jds.2016-11662.

Youden, W. J. 1950. Index for rating diagnostic tests. Cancer 3:32–35. https://doi.org/10.1002/1097-0142(1950)3:1<32::aid-cncr2820030106>3.0.co;2-3.

Zwald, N. R., K. A. Weigel, Y. M. Chang, R. D. Welper, and J. S. Clay. 2006. Genetic analysis of clinical mastitis data from on-farm management software using threshold models. J. Dairy Sci. 89:330–336. https://doi.org/10.3168/jds.S0022-0302(06)72098-7.

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