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Using milk leukocyte differentials for diagnosis of subclinical bovine mastitis

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This research study aimed to evaluate the use of the milk leukocyte differential (MLD) to: (a) identify quarter milks that are culture-positive; and (b) characterize the milk leukocyte responses to specific groups of pathogens causing subclinical mastitis. The MLD measures the absolute number and relative percentage of inflammatory cells in milk samples. Using the MLD in two dairy herds (170 and 172 lactating cows, respectively), we studied all lactating cows with a most recent monthly Dairy Herd Improvement Association somatic cell count (SCC) $>200 \times 10^3$ cells/ml. Quarter milk samples from 78 cows meeting study criteria were analysed by MLD and aseptically collected milk samples were subjected to microbiological culture (MC). Based upon automated instrument evaluation of the number and percentage of inflammatory cells in milk, samples were designated as either MLD-positive or – negative for subclinicial mastitis. Positive MC were obtained from 102/156 (65·4%) of MLD-positive milk samples, and 28/135 (20·7%) of MLD-negative milk samples were MC-positive. When MC was considered the gold standard for mastitis diagnosis, the calculated diagnostic *Se* of the MLD was 65·4% ($IC_{95\%}$ = 57·4 to 72·8%) and the *Sp* was 79·3% ($IC_{95\%}$ = 71·4 to 85·7%). Quarter milks positive on MC had higher absolute numbers of neutrophils, lymphocytes and macrophages, with higher neutrophils% and lymphocytes% but lower macrophages%. The Log_{10} (N/L) ratios were the most useful ratio to differentiate specific subclinical mastitis quarters from healthy quarters. Use of the MLD on cows with monthly composite SCC > 200×10^3 cells/ml for screening at quarter level identified quarters more likely to be culture-positive.

In conclusion, the MLD can provide an analysis of mammary quarter status more detailed than provided by SCC alone; however, the MLD response to subclinical mastitis was not found useful to specifically identify the causative pathogen.

Keywords: mastitis, diagnosis, milk leukocyte, differential cell count.

Subclinical mastitis (SM) is a common and economically significant disease of dairy cows, causing increased somatic cell counts (SCC) and decreased quality and yield of milk (Wilson et al. 1997; Pitkala et al. 2004; Halasa et al. 2009). Approximately 70 to 80% of mastitis losses are due to subclinical mastitis (Reneau & Packard, 1991). Subclinical mastitis infections are not evident and can persist in the mammary tissue throughout lactation (Pilla et al. 2013).

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Subclinical mastitis is most commonly diagnosed by microbial culture-based (MC) methods or SCC, which are both traditional and well-established tests for detection of subclinical mastitis (Oliver et al. 2004; Hand et al. 2012). Although SCC is a robust quantitative measurement, it does not differentiate cell types. Microbiological culture is based on collection of quarter milks aseptically for inoculation on culture medium and further testing for microorganism identification. The requirement for aseptic collection of milk samples for MC can be a disadvantage as the process is susceptible to contamination. Furthermore, traditional methods using MC can be labor-intensive and it may take up to 2–7 days to reach a diagnosis (Barreiro et al. 2010).

The milk leukocyte differential (MLD) has been investigated for potential in diagnosis of mastitis (Dulin et al. 1982; Kelly et al. 2000; Pillai et al. 2001; Dosogne et al. 2003; Schwarz et al. 2011a, b; Pilla et al. 2012, 2013). The MLD can detect changes in proportions of cell types in milk independently of the SCC, which could provide information about inflammatory processes in quarters otherwise considered healthy (Pilla et al. 2012). This information could be useful when control programs for milk pathogens are being applied (Pilla et al. 2013). The changes in cell ratio have been used for the identification of inflammatory processes in cows with low SCC, with the potential to differentiate milk from healthy quarters from those with early or late inflammation (Pilla et al. 2012). The MLD patterns of 6 out of 41 quarter milk samples with SCC values from ≥9000 to ≤46 000 cells/ml were described by Schwarz et al. (2011a) and their results revealed early inflammatory reactions based on the predominance of polymorphonuclear neutrophils (PMNL) (56–75%).

The MLD has been tested as an option to identify cows affected by any inflammatory process of the mammary gland, with the best results being reported by using logarithmic PMNL:lymphocyte ratio as the variable (Pilla et al. 2012). However, there is still little knowledge about the MLD and its application under field conditions. Therefore, the aims of this study were to evaluate the use of MLD to (a) identify quarter milks that are culture-positive; and (b) characterize the milk leukocyte responses to specific groups of pathogens causing subclinical mastitis.

Materials and methods

This research was approved by the North Carolina (NC) State University Institutional Animal Care and Use Committee (Raleigh).

Animals and herds

All lactating cows in herds A (170 cows) and B (172 cows) included in the most recent monthly Dairy Herd Improvement Association (DHIA) test results were screened for inclusion. From these cows, all lactating cows in each herd with a composite SCC > 200×10^3/ml, and with no history of clinical mastitis within the preceding month, were considered eligible for the study. The 78 selected cows included Holstein ($n = 52$), Jersey ($n = 19$) and cross bred cows ($n = 19$) in various lactations (1 to 7) and stages of lactation. Cows selected from the two NC dairy herds were subjected to detailed analysis of udder health status based on MLD and MC of aseptically collected quarter foremilk samples.

Cows on farms A (geometric mean bulk tank SCC = $112{\cdot}3 \times 10^3$ cells/ml) and B (geometric mean bulk tank SCC = $71{\cdot}3 \times 10^3$ cells/ml) were housed in free-stall and pack barn facilities, respectively, and were milked twice a day in parlors. Both herds had consistent application of mastitis control programs based on the recommendations of the National Mastitis Council (NMC; http://www.nmconline.org). In both herds, cows were fed a total mixed ration composed of corn silage, grain concentrate, and minerals, with access to hay. Water was available *ad libitum*. The farms were conventional milk producers with average milk yields of 9,015 (farm A) and 11,788 (farm B) kg/year per cow, respectively.

Milk sampling

Milk samples were collected from all functional quarters of the 78 eligible cows on the 2 farms. A total of 10 of the 312 possible quarters were non-functional. This left 302 quarter foremilk samples which were collected for MC according to NMC guidelines (Oliver et al. 2004). Before milking, teat ends were scrubbed with 70% isopropanol and the first three squirts of milk were discarded. Ten milliliters of milk per mammary quarter were collected aseptically. After the foremilk sampling for MC, milk from each quarter was collected into a quarter-based sampling chamber (Advanced Animal Diagnostics Company, AAD, Inc., Durham, NC) for MLD analysis. Quarter foremilk samples for MC were refrigerated (4–7 °C) until further analysis.

Microbiologic analysis

All microorganisms were isolated and categorized using procedures consistent with those recommended by the NMC (Oliver et al. 2004). Milk samples were plated within 24 h of collection. Milk samples were mixed and 0·1 ml of milk was inoculated onto trypticase soy agar plates with 5% sheep blood (Becton, Dickinson and Co., Sparks, MD). Inverted plates were incubated aerobically at 36 °C for 48 h and results were observed every 24 h regarding colony characteristics (shape, size, number, and color), haemolytic ability (presence and type), and possible contamination. Isolates were Gram stained and catalase reaction determined. Specific microbiology procedures are given in Supplementary File S1.

Somatic cell count

Monthly milk SCC were recorded from DHIA analysis, using composite milk samples with preservative (United DHIA, Radford, VA).

Milk leukocyte differential

Milk leukocyte differentials were determined on fresh milk collected within 15 days after the most recent DHIA test day. The instrument (QScout MLD® test, Advanced Animal Diagnostics, Inc., Durham, NC) uses fluorescent microscopy technology to count and differentiate immune cells in milk and was initially validated, as described in Supplementary File S2. In addition to providing absolute values for each cell type (neutrophil, lymphocyte and macrophage), the

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total leukocyte count and percentage and total of each cell type were reported and also used in an index to produce a categorical quarter diagnosis of healthy vs. infected (see Supplementary File S2). Phagocyte counts were calculated as the sum of macrophages and neutrophils. Because of the wide variations found within the cell populations, we evaluated the ratio among phagocytic cell groups expressed as a logarithm of base 10 with the aim of identifying a marker that indicated whether the quarters were more likely to be healthy or infected. The results were expressed as Log_{10} [Neutrophils/Lymphocytes] (Log_{10} (N/L)) and Log_{10} [Phagocytes/Lymphocytes] (Log_{10} (P/L)), as described previously by Pilla et al. (2012).

In the current study, samples were processed in research mode (See Supplementary File S2) to increase accuracy of calculated differentials. The mid-lactation index was selected with the manufacturer-recommended threshold set at 7. The reader (QScout Farm Lab, Advanced Animal Diagnostics, Inc., Durham, NC) has programmable threshold levels that may be selected by the user. By changing thresholds, a user can weight results towards higher sensitivity or higher specificity. In order to assess performance at various thresholds, settings were evaluated for the index range of 1–12 to allow sensitivity and specificity vs MC to be evaluated at each threshold setting.

Subclinical mastitis definition

Mammary quarters were considered to have an intramammary infection (IMI) when quarter milk samples showed isolation of significant bacterial colony numbers as described by Arruda et al. (2013), with slight modification. Since we plated 0·1 ml of milk, we considered presence of IMI as detection of any pathogen at any level, similar to that described by Dohoo et al. (2011).

Quarters selected from cows with SCC > 200×10^3 cells/ml were categorized at quarter level according the following criteria as previously described (DVG, 2002; Bansal et al. 2005): (a) healthy: culture-negative and total leukocyte count (TLC) ≤ 100×10^3 cells/ml; (b) latent subclinical mastitis (latent-SM): culture-positive and TLC ≤ 100×10^3 cells/ml; (c) nonspecific subclinical mastitis (nonspecific-SM): culture-negative and TLC > 100×10^3 cells/ml; and (d) specific subclinical mastitis (specific-SM): culture-positive and TLC > 100×10^3 cells/ml.

Experimental design and statistical analysis

Data are presented as means ± SE. Associations between the MLD and MC status of the udder quarters were analyzed by applying linear mixed models with the SAS® program (version 9.3; SAS Institute Inc., Cary, NC, USA) after testing for residual normality and homogeneity of variance. We included data from all foremilk samples without contamination and with complete results for MLD and MC. The statistical model included the fixed effects of herd, cow, position of the udder quarter, milk yield, lactation number, parity number, breed and IMI. Statistical significance was defined at *P*-value <0·05. The statistical model used in analysis is given in Supplementary File S3.

The analysis was performed on Log_{10} transformation for SCC, absolute values for each cell type, the total leukocyte count and percentage and total of each cell type to provide normal distribution of the data. Data were anti-log_{10} transformed for presentation of the results and discussion. The MLD sensitivity (*Se*) and specificity (*Sp*) were determined comparing the MC as a standard methodology with the categorical quarter diagnosis from MLD technology (healthy vs infected) using on-line statistical software (MedCalc for Windows, version 16·8, MedCalc Statistical Software, 2016).

Results

Microbiologic analysis

A total of 302 quarter milk samples was aseptically collected from eligible quarters. There were 8 contaminated samples, leaving 294 quarter samples with usable culture results. Frequency of mastitis pathogen identification by MC of 294 quarter samples is given in Table 1. Overall, 130 quarters (44·2%) were classified as culture-positive and 164 (55·8%) were negative on culture. Minor pathogens (n = 50) accounted for 17·0% of total samples, being composed of CNS (n = 38) and *Corynebacterium* spp. (n = 12). Coagulase negative staphylococci (CNS) were the most commonly isolated mastitis-causing pathogen. Among the CNS group, *Staphylococcus chromogenes* was the most frequent, being found in 24/38 CNS isolates (8·2% of all samples). A variety of other CNS species was found in the remainder (Table 1).

Major pathogens were identified in 68 quarters (23·1% of all samples: Table 1). Out of these, 37 quarters had isolation of contagious pathogens, all *S. aureus* (12·6%). There were 31 quarters with environmental pathogens (10·5%), primarily streptococci. A total of 12 quarters (4·1%) were identified as positive for other miscellaneous pathogens (*Nocardia* spp., yeast and *Prototheca* spp.).

Somatic cell count and milk leukocyte differential

Comparison of MLD results for quarters with variable mastitis definitions (healthy, latent-SM, non-specific-SM and specific-SM). There were 102 mammary quarters classified as healthy (35%), 32 as latent-SM (11%), 59 as nonspecific-SM (20·3%) and 98 as specific-SM (33·7%: Table 2). Mammary quarters with specific-SM ($772{\cdot}5 \times 10^3$ cells/ml), nonspecific-SM ($527{\cdot}1 \times 10^3$ cells/ml) and latent-SM ($40{\cdot}6 \times 10^3$ cells/ml) had higher TLC than healthy quarters ($25{\cdot}1 \times 10^3$ cells/ml). The neutrophils% were greater in specific-SM cases (65·7%) than nonspecific-SM cases (55·2%), latent-SM cases (55·0%) and healthy quarters (49·4%). Therefore, healthy quarters had the lowest mean value of absolute number of neutrophils ($12{\cdot}3 \times 10^3$ cells/ml). Although mammary quarters with latent-SM, nonspecific-SM

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Table 1. Frequency of mastitis pathogen identification by microbiological culture of mammary quarter foremilk samples ($n = 294$) from two herds in North Carolina

	Mammary quarter foremilk samples				
	No. isolates by Farm				
Microorganisms	A	B	Total	Absolute frequency (%)	Relative frequency (%)
No.	202	92	294	100·00	–
Negative culture	110	54	164	55·78	–
Positive culture	92	38	130	44·22	100·00
Minor pathogens:	32	18	50	17·01	38·46
Coagulase negative Staphylococci	23	15	38	12·93	29·23
S. chromogenes	12	12	24	8·16	18·46
Other coagulase negative staph[†]	11	3	14	4·77	10·77
Corynebacterium spp.	9	3	12	4·08	9·23
Major pathogens:	51	17	68	23·1	52·3
Contagious pathogens—all *Staphylococcus aureus*	25	12	37	12·59	28·46
Environmental pathogens	26	5	31	10·54	23·85
Streptococcus spp.[‡]	19	2	21	7·14	16·15
Enterococcus spp.[§]	5	1	6	2·04	4·62
Enterobacter cloacae	0	1	1	0·34	0·77
Escherichia coli	1	0	1	0·34	0·77
Serratia marcescens	0	1	1	0·34	0·77
Non-fermenter species	1	0	1	0·34	0·77
Miscellaneous pathogens:	9	3	12	4·08	9·23
Nocardia spp.	5	0	5	1·70	3·85
Yeast	3	2	5	1·70	3·85
Prototheca spp.	1	1	2	0·68	1·54

†Other coagulase-negative staphylococci for Farms A and B, respectively, included *S. capitis* (0 and 1), *S. hominis* (1 and 0), *S. hyicus* (2 and 1), *S. lugdnensis* (2 and 0), *S. sciuri* (1 and 0), *S. xylosus* (1 and 0), and other staphylococci (4 and 1)

‡Streptococci isolated from Farms A and B, respectively, included *Str. bovis* (2 and 0), *Str. dysgalactiae* (4 and 0), *Str. uberis* (12 and 2), and *Aerococcus viridans* (1 and 0)

§Enterococci isolated on farms A and B, respectively, included *Ent. avium* (1 and 0), *Ent. durans* (0 and 1), *Ent. faecium* (2 and 0), and other enterococci (2 and 0)

and specific-SM had higher TLC than healthy quarters, the macrophages% were lower in quarters with specific-SM (12·3%), nonspecific-SM (17·3%) and latent-SM (23·0%), when compared to healthy quarters (28·9%). The lymphocytes% and phagocytes% were similar among tested groups, but mammary quarters with specific-SM, nonspecific-SM and latent-SM had higher mean value of absolute number of lymphocytes and phagocytes than healthy quarters (Table 2). We evaluated the ratio among phagocytic cell groups expressed as a logarithm of base 10 aiming to identify cows more likely to be milk culture-positive according to our definition of mastitis. We found that the cell ratio Log_{10} (N/L) was higher in quarters with specific-SM (0·57), nonspecific-SM (0·50) and latent-SM (0·49) than healthy quarters (0·44). Using the cell ratio Log_{10} (N/L) would provide some differentiation of the quarters classified according to our mastitis definition. On the other hand, there was no difference of the cell ratio Log_{10} (P/L) between quarters with specific-SM (0·66), nonspecific-SM (0·66), latent-SM (0·67) and healthy quarters (0·67) (Table 2).

No influence of quarter position, milk yield, parity and breed could be found on milk MLD results. However, we observed an effect of stage of lactation ($DIM_1 = 4$ to 100, $DIM_2 = 101$ to 200 and $DIM_3 = 201$ to 431) on milk MLD results. The greater the DIM, the greater the macrophages % ($P < 0·04$) and the phagocytes% ($P < 0·01$), but lower the lymphocytes% ($P < 0·01$) (Fig. 1).

Performance of categorical analysis on instrument readout (negative or healthy vs positive or infected). A total of 294 quarter samples were submitted to the automated technology based upon readout results. Three quarter milk samples were reported as disabled by the MLD automated technology, leaving results available for a total of 291 quarters (Table 2). Out of 102 mammary quarters designated as healthy, 98 quarters (96·1%) were categorized as negative and 4 (3·9%) as positive by the automated technology based upon readout results. A total of 32 quarters were classified as latent-SM, with 26 quarters (81·3%) classified negative and 6 as a positive. There were 59 quarters designated

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Table 2. Mean values for individual cell populations and combinations of cell populations from quarter milk samples considering the mastitis definition ($n = 291$)

Cell population[a]	Healthy	Latent	Non-specific subclinical mastitis	Specific subclinical mastitis
All quarters tested, no. (%)	102 (35)	32 (11)	59 (20·3)	98 (33·7)
Equipment results, no. (%)				
Negative (135)	98 (96·1)	26 (81·3)	9 (15·3)	2 (2)
Positive (156)	4 (3·9)	6 (18·7)	50 (84·7)	96 (98)
SCC† X 1,000/ml	570·61 ± 148[a]	705·72 ± 302·24[a]	561·72 ± 103·66[a]	701·87 ± 164·81[a]
TLC‡ X1,000/ml	4·40 ± 0·05 (25·12)[d]	4·61 ± 0·08 (40·63)[c]	5·72 ± 0·06 (527·11)[b]	5·89 ± 0·05 (772·50)[a]
Neutrophils%	4·69 ± 0·02 (49·37)[c]	4·74 ± 0·02 (54·99)[b]	4·74 ± 0·02 (55·16)[b]	4·82 ± 0·02 (65·72)[a]
Neutrophils X1,000/ml	4·09 ± 0·05 (12·34)[d]	4·35 ± 0·09 (22·49)[c]	5·46 ± 0·07 (291·61)[b]	5·71 ± 0·06 (510·15)[a]
Macrophages%	4·46 ± 0·04 (28·93)[a]	4·36 ± 0·05 (23·03)[b]	4·24 ± 0·04 (17·29)[c]	4·09 ± 0·04 (12·29)[d]
Macrophages X1,000/ml	3·87 ± 0·05 (7·36)[b]	3·95 ± 0·08(8·91)[b]	4·95 ± 0·06 (90·07)[a]	4·98 ± 0·05 (96·58)[a]
Lymphocytes%	4·25 ± 0·03 (17·71)[a]	4·25 ± 0·04 (17·79)[a]	4·24 ± 0·03 (17·30)[a]	4·25 ± 0·03 (17·89)[a]
Lymphocytes X1,000/ml	3·66 ± 0·06 (4·53)[d]	3·86 ± 0·09 (7·23)[c]	4·96 ± 0·07 (91·01)[b]	5·14 ± 0·06 (138·39)[a]
Phagocytes%	4·92 ± 0·01 (82·87)[a]	4·92 ± 0·02 (82·64)[a]	4·90 ± 0·02 (79·41)[a]	4·91 ± 0·01 (82·07)[a]
Phagocytes§ X1,000/ml	4·32 ± 0·05 (20·73)[d]	4·53 ± 0·08 (33·54)[c]	5·62 ± 0·06 (420·15)[b]	5·80 ± 0·05 (631·98)[a]
Log_{10} (N/L)¶	0·44 ± 0·04[b,c]	0·49 ± 0·05[a,b]	0·50 ± 0·04[a,b]	0·57 ± 0·04[a]
Log_{10} (P/L)‡‡	0·67 ± 0·04[a]	0·67 ± 0·05[a]	0·66 ± 0·05[a]	0·66 ± 0·04[a]

[a]Cells were presented as absolute number and in ratio
[a]Data are presented as means on Log_{10} transformation ± SE
[a]Data are presented as means on antiLog$_{10}$ transformation between parentheses
[a]Different letters within row were significantly different ($P < 0·05$)
†Geometric mean of somatic cell count at cow level from most recent DHIA test day prior to quarter sample collection
‡Total Leukocyte Count and other measures on quarter basis
§Phagocyte count were based on the sum of macrophages and neutrophils
¶Log_{10} (N/L) = Log_{10} [Neutrophils/Lymphocytes]
‡‡Log_{10} (P/L) = Log_{10} [Phagocytes/Lymphocytes]

as having nonspecific SM, and the automated technology categorized 9 quarters as negative (15·3%) and 50 (84·7) as positive. Ninety-eight mammary quarters with specific-SM cases were categorized as 2 negative (2%) and as 96 (98%) as positive.

As shown in Table 2, the MLD categorized 156 mammary quarters as positive (53·6%) and 135 as a negative (46·4%). Out of all quarters categorized as negative ($n = 135$) by the MLD, 79·3% had negative cultures ($n = 107$) and 20·7% had positive cultures ($n = 28$). On the other hand, out of all quarters considered positive ($n = 156$) by the automated technology, 65·4% had positive cultures ($n = 102$) and 34·6% had negative cultures ($n = 54$). When MC was considered the gold standard for mastitis diagnosis, the calculated diagnostic *Se* of the MLD was 65·4% ($IC_{95\%} = 57·4$ to 72·8%) and the *Sp* was 79·3% ($IC_{95\%} = 71·4\%$ to 85·7%). Using MC results as the 'gold standard,' *Se* and *Sp* of the categorical instrument readout results (healthy or infected) based upon cut-offs ranging from 1–12 are shown in Fig. 2. Sensitivity progressively increased from a minimum of 50·4% at a user setting of 1 to a maximum of 71·3% at a setting of 12 (Fig. 2). Specificity progressively decreased from a maximum of 86·7% at user setting 1 to 66·7% at setting 12 (Fig. 2).

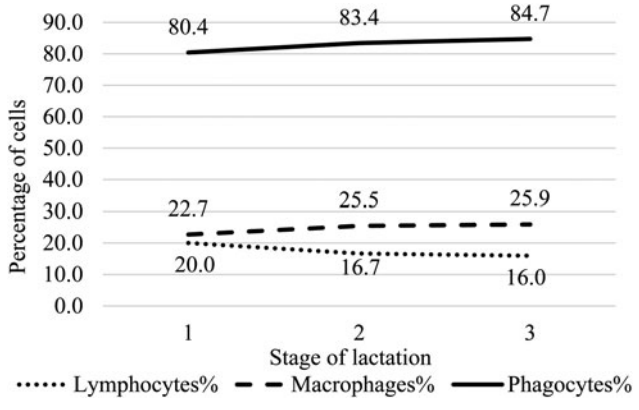

Fig. 1. Effect of stage of lactation ($DIM_1 = 4$ to 100, $DIM_2 = 101$ to 200 and $DIM_3 = 201$ to 431) on milk macrophages%, lymphocytes % and phagocytes%.

Comparison of MLD results for quarters following categorization by mastitis pathogen groups (minor, environmental, contagious and miscellaneous). A total of 161 healthy quarters (culture-negative) were selected and compared to 130 infected quarters (culture-positive) according to the pathogen category. The MC and MLD results from mammary quarters infected with minor pathogens ($n = 50$; 17%), environmental pathogens ($n = 31$; 10·5%), contagious pathogens ($n = 37$; 12·6%), and miscellaneous pathogens ($n = 12$; 4·1%) were compared to healthy quarters (Table 3).

Mammary quarters subclinically infected by miscellaneous ($992·4 \times 10^3$ cells/ml) and contagious ($973·6 \times 10^3$

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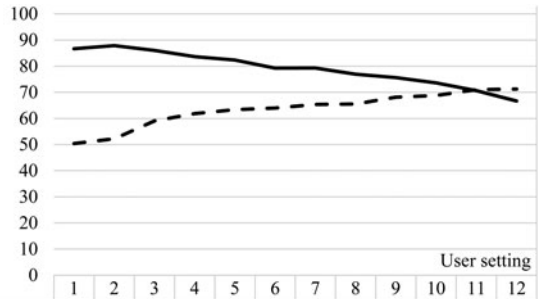

	1	2	3	4	5	6	7	8	9	10	11	12
Sensitivity	50.4	52.2	59.1	61.9	63.4	64	65.4	65.5	68.2	68.8	71	71.3
Specificity	86.7	87.9	86	83.6	82.4	79.2	79.3	76.9	75.6	73.6	70.7	66.7

Fig. 2. Sensitivity and specificity evaluated at different threshold setting of QScout® MLD.

cells/ml) pathogens had a similar TLC, but both group of pathogens had higher TLC than healthy quarters ($76{\cdot}3 \times 10^3$ cells/ml) and quarters infected by environmental ($332{\cdot}2 \times 10^3$ cells/ml) and minor pathogens ($134{\cdot}4 \times 10^3$ cells/ml). Mammary quarters subclinically positive with miscellaneous, contagious, environmental and minor pathogens had higher mean values of absolute number of neutrophils and neutrophils% than healthy quarters. The absolute number of macrophages was higher in all infected quarters as compared to healthy quarters, however, the % macrophages was higher in healthy quarters than quarters infected by any pathogen. This represented a proportional decrease of macrophages% but increase of neutrophils% when a quarter became infected. The lymphocytes% and phagocytes% were similar among tested groups, but mammary quarters infected by any pathogen had higher mean numbers of lymphocytes and phagocytes as compared to healthy quarters (Table 3). The cell ratio Log_{10} (N/L) was significantly higher in quarters infected by miscellaneous (0·62), contagious (0·57), environmental (0·53) and minor pathogens (0·52) than in healthy contralateral quarters (0·47). On the other hand, there was no difference in the cell ratio Log_{10} (P/L) between healthy quarters (0·67) and quarters infected with miscellaneous (0·69), contagious (0·67), environmental (0·66) and minor pathogens (0·65) (Table 3).

Discussion

It has been proposed that the MLD can identify changes in relative cell populations before the increase in TLC occurs in the course of inflammatory process (Pilla et al., 2012, 2013). Based upon this, we asked if the use of MLD would be able to (a) identify quarter milks more likely to be culture-positive; and (b) characterize the milk leukocyte responses to specific groups of pathogens causing subclinical mastitis. We found that 65·4% of quarters producing MLD-positive test results were positive for MC, while 20·7% of quarters testing MLD-negative were culture-positive. The Log_{10} (N/L) ratios were shown to be the most useful ratio to differentiate specific subclinical mastitis cases from healthy quarters. In addition to giving a total cell count, the MLD can be used for more detailed evaluation of udder health status.

Microbiologic analysis

Both farms used for this study were representative of smaller farms with mastitis problems warranting investigation, in that a considerable number of various pathogens were detected including *Staphylococcus aureus*. The validity of using elevated composite cow SCC ($>200 \times 10^3$ cells/ml in most recent test) as a criterion for selection was affirmed by the finding that an average of 44·2% of quarter samples tested produced a positive microbiological result (45·6% for farm A and 41·3% for farm B). Both farms had approximately the same profile of pathogens. We have found *Staphylococcus aureus* as a frequent problem in some dairies in our region. The CNS were frequently isolated, similar to other studies (e.g., Makovec & Ruegg (2003); Tomazi et al. (2015)). Considering all isolates, there were 38·5% minor pathogens, with CNS predominating, 28·5% *Staphylococcus aureus*, 23·8% environmental pathogens with streptococci predominating, and 9·2% infrequent pathogens such as *Nocardia* spp., yeasts and *Prototheca* spp. The profile of pathogens found in positive cultures makes the herds used appropriate for an investigation of mastitis diagnostics considering multiple etiologies.

Somatic cell count and milk leukocyte differential

Comparison of MLD results for quarters with variable mastitis definition (healthy, latent-SM, non-specific-SM and specific-SM). The significantly higher TLC for specific-SM ($772{\cdot}5 \times 10^3$ cells/ml) vs. healthy quarters ($25{\cdot}1 \times 10^3$ cells/ml) samples was not surprising, as it was part of the selection criteria. The magnitude of the difference, as well as the significantly higher total neutrophils, total macrophages, total lymphocytes, and total phagocytes was consistent with expectations, similar to other studies (Pillai et al. 2001; Dosogne et al. 2003; Schwarz et al. 2011a, b; Pilla et al. 2012, 2013). Schwarz et al. (2011a) showed that PMNL in milk samples with SCC values $<6{\cdot}25 \times 10^3$ cells/ml were rare (mean proportion = 15%). Pillai et al. (2001) evaluated the MLD from mammary quarters with high SCC ($>250 \times 10^3$ cells/ml) in comparison to the quarters with low SCC ($<250 \times 10^3$ cells/ml), and they observed that the TLC and PMNL were consistently higher in quarters with high SCC. Additionally, it was reported that quarters with high SCC, TLC and PMNL were more often positive on MC (62 to 87%) compared with those with low SCC, TLC and PMNL (37 to 51%).

Similar to our study, Pillai et al. (2001) observed that 33 to 49% (mean = 40%) of the inflammatory cells from infected quarters were PMNL, while PMNL constituted only 17 to 25% (mean = 20%) of the inflammatory cells counted from uninfected quarters. Schwarz et al. (2011b) observed that

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Table 3. Mean values for individual cell populations and combinations of cell populations from quarter milk samples considering the category of pathogens subclinical mastitis-causing ($n = 291$)

Cell population[a]	Healthy	Minor	Environmental	Contagious	Miscellaneous
All quarters tested, no. (%)	161 (55·3)	50 (17·2)	31 (10·7)	37 (12·7)	12 (4·1)
Equipment results, no. (%)					
Negative	107 (65·2)	17 (34)	6 (19·4)	4 (10·8)	1 (8·3)
Positive	54 (34·8)	33 (66)	25 (80·6)	33 (89·2)	11 (91·7)
SCC† X1,000/ml	577·47 ± 99·49[a]	587·16 ± 192·11[a]	1151·93 ± 487·43[a]	630·59 ± 80·43[a]	684·47 ± 232·21[a]
TLC‡ X1,000/ml	4·88 ± 0·08 (76·37)[e]	5·13 ± 0·12 (134·40)[d]	5·52 ± 0·14 (332·20)[c]	5·99 ± 0·13 (973·64)[a,b]	6·00 ± 0·21 (992·43)[a]
Neutrophils%	4·71 ± 0·01 (51·57)[e]	4·77 ± 0·02 (58·99)[c,d]	4·79 ± 0·02 (61·08)[b,c]	4·82 ± 0·02 (66·31)[a,b]	4·86 ± 0·03 (71·99)[a]
Neutrophils X1,000/ml	4·59 ± 0·09 (39·24)[e]	4·91 ± 0·13 (80·82)[d]	5·32 ± 0·16 (207·87)[c]	5·81 ± 0·14 (648·04)[a,b]	5·85 ± 0·23 (706·32)[a]
Macrophages%	4·38 ± 0·04 (23·91)[a]	4·20 ± 0·05 (15·83)[b]	4·19 ± 0·06 (15·35)[b]	4·09 ± 0·05 (12·34)[b]	4·10 ± 0·08 (12·70)[b]
Macrophages X1,000/ml	4·27 ± 0·10 (18·43)[d,e]	4·33 ± 0·12 (21·25)[d]	4·70 ± 0·14 (50·47)[c]	5·10 ± 0·13 (126·27)[a,b]	5·15 ± 0·19 (142·40)[a]
Lymphocytes%	4·24 ± 0·03 (17·55)[a]	4·26 ± 0·04 (18·06)[a]	4·26 ± 0·04 (18·11)[a]	4·25 ± 0·04 (17·73)[a]	4·23 ± 0·06 (17·15)[a]
Lymphocytes X1,000/ml	4·14 ± 0·07 (13·86)[e]	4·39 ± 0·12 (24·46)[d]	4·78 ± 0·14 (59·91)[c]	5·23 ± 0·12 (171·44)[a,b]	5·23 ± 0·22 (171·20)[a]
Phagocytes%	4·91 ± 0·01 (81·60)[a]	4·91 ± 0·02 (81·10)[a]	4·92 ± 0·02 (82·64)[a]	4·92 ± 0·02 (82·55)[a]	4·93 ± 0·02 (84·61)[a]
Phagocytes§ X1,000/ml	4·79 ± 0·09 (62·33)[e]	5·04 ± 0·13 (108·84)[d]	5·44 ± 0·15 (275·74)[c]	5·91 ± 0·14 (807·98)[a,b]	5·93 ± 0·22 (849·77)[a]
Log_{10} (N/L)¶	0·47 ± 0·04[b]	0·52 ± 0·05[a,b]	0·53 ± 0·05[a,b]	0·57 ± 0·05[a]	0·62 ± 0·07[a]
Log_{10} (P/L)††	0·67 ± 0·04[a]	0·65 ± 0·05[a]	0·66 ± 0·05[a]	0·67 ± 0·05[a]	0·69 ± 0·07[a]

[a]Cells were presented as absolute number and in ratio
[a]Data are presented as means on Log_{10} transformation ± SE
[a]Data are presented as means on antiLog_{10} transformation between parentheses
[a]Different letters within row were significantly different ($P < 0·05$)
†Geometric mean of somatic cell count at cow level from most recent DHIA test day prior to quarter sample collection
‡Total Leukocyte Count and other measures on quarter basis
§Phagocyte count were based on the sum of macrophages and neutrophils
¶ Log_{10} (N/L) = Log_{10} [Neutrophils/Lymphocytes]
†† Log_{10} (P/L) = Log_{10} [Phagocytes/Lymphocytes]

PMNL were the dominant cell population in milk samples of diseased quarters, with proportions of PMNL ≥ 65%.

Results of our study are most comparable to those of Pilla et al. (2012), who compared differential cell counts from 96 normal quarters with 92 abnormal quarters categorized as latent mastitis, unspecific mastitis and subclinical mastitis. Similar to our findings, Pilla et al. (2012) found that lymphocytes, neutrophils, and Log_{10} (N/L) were significantly higher in abnormal quarters. Macrophages were not significantly affected in the study of Pilla et al. (2012).

Our numerical results for neutrophils% were very similar to those reported by Pilla et al. (2012). Although we detected differences in macrophages% between quarters with specific-SM vs. those with nonspecific-SM, latent-SM and healthy quarters, the absolute values we obtained for macrophages% were very similar to those of Pilla et al. (2012). In general, in the present study there was a proportional decrease of macrophages% with increases of neutrophils% when the quarter became infected. This result was similar to those described by Schwarz et al. (2011b), who reported a significant negative correlation between macrophage% and SCC.

In our study, in which we classified the mammary quarters in a slightly different manner, Log_{10} (N/L) mean values from healthy quarters (0·44) were significantly lower than the latent-SM (0·49), nonspecific-SM (0·50) and specific-SM (0·57) groups (Table 2). These mean values of Log_{10} (N/L) were lower than what Pilla et al. (2012) reported. Pilla et al. (2012) categorized quarters in four groups (healthy quarters, latent mastitis-LM, nonspecific mastitis-UM and subclinical mastitis-SM) according to the SCC and MC results. They found that the Log_{10} (PMNL/Lymphocytes) mean values in healthy quarters (0·11) were significantly lower than those in groups with latent mastitis (0·57), nonspecific mastitis (0·73), and subclinical mastitis (0·94). Similar to our study, Log_{10} (N/L) was significantly different in quarters with specific-SM (0·57) vs. healthy quarters (0·44), but not for Log_{10} (P/L), indicating the merit of investigating quarters with Log_{10} (N/L) > 0·44 because they may be more often infected. This value is similar to what Pilla et al. (2013) reported as a cutoff value. This categorization of the quarters in different types of mastitis is important since it may minimize the effect of positive and false negatives, as an example the nonspecific-SM cases (even in absence of bacteria has high SCC).

According to Pilla et al. (2013), no influence of sampling day, parity, lactation stage, or quarter position could be found on leukocyte differential results. We did not observe a similar finding based on our results, because the greater the DIM the greater the macrophages% and the phagocytes%, but lower the lymphocytes% which is in agreement with previous results (Dosogne et al. 2003).

Performance of categorical analysis on readout (negative or healthy vs. positive or infected). The MLD readout results corresponded reasonably well with the quarter culture results, with 79·3% of negative MLD results being negative on culture, while 65·4% of MLD-positive quarters were culture-positive. Our reported *Se* of 65·4% and *Sp* of 79·3% were similar to those reported in prior studies. Pilla et al. (2013) reported *Se* of 73·3% and *Sp* of 73·6%. Adjustment of user settings from 1 to 12 would allow user optimization of settings. Sensitivities progressively increased from 50·4% at setting 1 to 71·3% at setting 12, while specificities decreased from 86·7% at setting 1 to 66·7% at setting 12 (Fig. 2).

Comparison of MLD results for quarters following categorization by mastitis pathogen groups (minor, environmental, contagious and miscellaneous). The difference in TLC was striking when quarters infected by any pathogen were compared to healthy quarters, as was the difference in neutrophils%, macrophages% and Log_{10} (N/L). Schwarz et al. (2011a) described results similar to our study, reporting significant differences of cellular components in milk between quarters infected with pathogens as compared to healthy quarters. Although we have observed differences of MLD between quarters infected by any pathogen vs. healthy quarters, we found that the MLD in response to SM cannot be used to specifically identify the causative pathogen.

One purpose of our study was to consider the actual field application of this technology. Milk culture or other forms of microbiological analysis can be costly to the producer. An obvious use of the MLD would be to focus on cows with monthly SCC above some cut-off point (here, $>200 \times 10^3$ cells/ml) with screening the infection at quarter level by providing a more rapid diagnosis performed by automated technology based upon 'on-farm differential cells' readout results.

Two recent studies (Hockett et al, 2014a, b) have evaluated the automated technology readout results for selective dry cow therapy after diagnosis of infection by MLD compared to blanket dry cow treatment with cephapirin benzathine and cloxacillin. These studies from Hockett et al. (2014a, b) indicated that the use of MLD to guide selective treatment of infected cows reduced the use of cephapirin benzathine (47%) and cloxacillin (58%), and resulted in similar rate of infection, SCC and milk compared to blanket antibiotic therapy.

Conclusion

The MLD response to subclinical mastitis can provide more detailed diagnostic evaluation of than provided by SCC alone. We found that 65·4% of quarters producing MLD-positive test results were positive for MC, with 20·7% of quarters testing MLD-negative found as culture-positive. Similar to other previous studies, quarters positive on culture had higher absolute numbers of neutrophils, lymphocytes and macrophages, with higher neutrophils% and lymphocytes% but lower macrophages%. The Log_{10} (N/L) ratios were shown to be the most useful ratio to differentiate specific subclinical mastitis cases from healthy quarters. An

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obvious use of the MLD would be to help focus on the cows with monthly SCC above some limit (here >200 × 10^3 cells/ml) for screening the infection at quarter level by providing a more rapid diagnosis performed by automated technology based upon 'on-farm differential cells' readout results. Although MLD was able to identify quarters more likely to be milk culture-positive it was not found useful to help identify the specific causative pathogen.

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Supplementary material

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